Biochimica et Biophysica Acta, 593 (1980) 441—453

BBA 47977

RESONANCE RAMAN SPECTROSCOPY OF CHEMICALLY MODIFIED AND ISOTOPICALLY LABELLED PURPLE MEMBRANES

I. A CRITICAL EXAMINATION OF THE CARBON-NITROGEN VIBRATIONAL MODES

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(Received July 22nd, 1980)

Key words: Resonance Raman spectroscopy; Purple membrane; Bacteriorhodopsin; Chemical modification; Retinal

Summary

Resonance Raman spectra of bacteriorhodopsin are compared to the spectra of this protein modified in the following ways: (1) selective deuteration at the C-15 carbon atom of retinal, (2) full deuteration of the retinal, (3) the addition of a conjugated double bond in the β-ionone ring (3-dehydroretinal), (4) full deuteration of the protein and lipid components, (5) ^{15}N enrichment of the entire membrane and (6) deuteration of the entire membrane (including the retinal). A detailed comparison of the ^{15}N-enriched membrane and naturally occurring purple membrane from 800 cm^{-1} to 1700 cm^{-1} reveals that ^{15}N enrichment affects the frequency of only two vibrational modes. These occur at 1642 cm^{-1} and 1620 cm^{-1} in naturally occurring purple membrane and at 1628 cm^{-1} and 1615 cm^{-1} in the ^{15}N-enriched samples. Therefore, this pair of bands reflects the states of protonation of the Schiff base. However, our data also indicate that neither of these modes are simple, localized $C=\overset{\oplus}{N}-H$ or C=N stretching vibrations. In the case of the 1642 cm^{-1} band motions of the retinal chain beyond C-15 are not significantly involved. On the other hand, in the 1620 cm^{-1} band atomic motions in the isoprenoid chain beyond C-15 are involved.

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Abbreviation: BR, bacteriorhodopsin.

Introduction

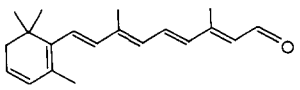
The molecular mechanism by which membrane-bound ion pumps function is a question of considerable interest in cell biology. Bacteriorhodopsin is a well characterized proton pump [1,2], which is found in purple membrane patches in the plasma membrane of the halophile *Halobacterium halobium* [3] and can be excited directly by light absorption [1,2]. Resonance Raman spectroscopy has proven itself to be a unique probe of the active site of this membrane-bound ion pump [4—12]. Initial experiments in our laboratory [5] demonstrated that two vibrational modes appeared in the region where Schiff-base carbon-nitrogen stretching vibrations were observed. One of these vibrational modes at 1642 cm^{-1} could be associated with the initial unexcited state of the protein (BR_{570}), and suspension in ^2H_2O demonstrated that this mode included contributions from the $C=\overset{\oplus}{N}$-H stretch. Thus, based on these data, Lewis et al. [5] concluded that the retinal chromophore in BR_{570} was complexed through a protonated Schiff-base linkage to the protein.

As a result of the photochemistry, BR_{570} disappears, and after several intermediate states, a pigment complex, M_{412}, is formed in microseconds [13,14]. In the investigation by Lewis et al. [5] discussed above, it was also shown that as this M_{412} intermediate was produced the 1642 cm^{-1} band decreased in intensity and a second vibrational mode appeared in this region at 1620 cm^{-1}. This vibrational mode was attributed to the unprotonated carbon-nitrogen stretch based on the close correspondence both in frequency and intensity of this band to carbon-nitrogen stretching vibrations in model compounds of unprotonated retinal Schiff bases. On the basis of these data it was concluded [5] that the Schiff base linkage deprotonates during the bacteriorhodopsin proton pumping cycle. However, in these early investigations [5], the conclusion that the 1620 cm^{-1} band does include contributions from the unprotonated carbon-nitrogen stretch was not directly verified using isotopic enrichment and/or chemical modification. The present paper addresses for the first time the assignment of the 1620 cm^{-1} band using chemically modified retinals, selectively deuterated retinals, fully deuterated retinals, ^{15}N-enriched protein and fully deuterated protein.

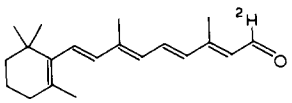
Materials and Methods

H. halobium cells (S-9) were grown by standard procedures and bacteriorhodopsin was isolated and purified by the method of Kanner and Racker [15]. Fully deuterated bacteriorhodopsin and ^{15}N-enriched bacteriorhodopsin were grown at Argonne National Laboratories [16]. Resonance Raman measurements have shown that the isotopic substitution was >95%. All the bacteriorhodopsin samples were treated with a 0.5% solution of deoxycholic acid to remove bacterial carotenoids. The purity was checked by the absence of the Raman lines at 1001, 1156 and 1515 cm^{-1}, characteristic for normal and ^{15}N-enriched bacteriorhodopsin and at 1300 and 1460 cm^{-1} for fully deuterated bacteriorhodopsin.

For reconstitution of retinal analogs, the apomembrane was obtained by the method of Oesterhelt and Schuhmann [17]. All-*trans*-3-dehydroretinal (I) was



I



II

a gift from Drs. V. Gloor and F. Weber from Hoffman-La Roche, Inc. All-*trans*-[15-^{2}H]retinal (II) was obtained by reduction of all-*trans*-retinal (Eastman-Kodak) with $LiAl^2H_4$ to the deuterated retinal followed immediately by oxidation with MnO_2 [18]. The product was purified by liquid chromatography and nuclear magnetic resonance spectra verified the absence of the C-15 proton [19]. The reconstitutions were performed by adding a concentrated (10^{-3} M) ethanol solution of the retinal analog to the suspension of the apomembrane (10^{-5} M), until no further increase was observed in the absorption band of the pigment.

Resonance Raman spectra were obtained by excitation with 50 mW of 457.9 nm light from an Ar^+ laser or 40 mW of 530.9 nm light from Kr^+ laser. The sample suspensions, having $A \simeq 1$ per cm at λ_{max}, were placed in capillary tubes and the scattering was collected at a right angle to the excitation beam, and focused onto a Spex 1401 monochromator. A cooled RCA C31034 photomultiplier tube and photon-counting electronics were used. All spectra were taken with 2 cm^{-1} resolution and monochromator step, and the counting time per step was 10—30 s.

Results and Discussion

In a previous resonance Raman investigation of the purple membrane from this laboratory [5] we unequivocally assigned the vibrational mode at 1642 cm^{-1} to the carbon-nitrogen stretching vibration of a protonated Schiff base linkage. In this same investigation we noted that a band at 1620 cm^{-1} appears when the M_{412} intermediate is present [5]. The extremely close correspondence of the frequency and intensity of this vibrational mode with the carbon-nitrogen stretching frequency in model unprotonated retinal Schiff bases was the basis for suggesting that M_{412} contains an unprotonated Schiff base linkage. Subsequent kinetic resonance Raman spectra indicated that the 1620 cm^{-1} vibrational mode could be detected before the appearance of the M_{412} C=C stretch at 1566 cm^{-1} [6]. This suggested that an additional unprotonated intermediate, X, may be present between L and M and the above suggestion was supported by the observation of a C=C stretch at 1552 cm^{-1} with a time evolution that also could not be related to L or M production [8]. However, none of these investigations addressed directly the assignment of the C=N vibrational mode in X or for that matter in M_{412}. In this study we have investigated a variety of isotopically and chemically modified purple membranes which not only bear directly on the assignment of the 1620 cm^{-1} band, but also result in a better definition of protein, retinal ring and retinal chain effects on the resonance

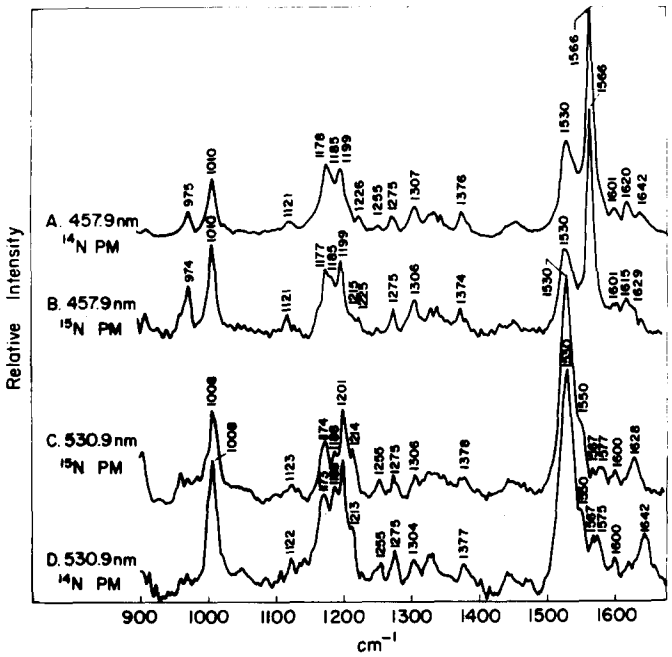


Fig. 1. Resonance Raman spectra of ^{14}N-labelled bacteriorhodopsin (A and D) and bacteriorhodopsin fully enriched with ^{15}N (B and C), obtained with 457.9 nm (A and B) or 530.9 nm (C and D) excitation. Laser powers were 50 mW, spectral resolution 2 cm^{-1} and counting time 10 s per channel. PM, purple membrane.

Raman spectra of purple membranes. The results will now be discussed in the following order: Fully enriched ^{15}N purple membranes, purple membranes reconstituted with retinal selectivity deuterated at C-15 and membranes reconstituted with 3-dehydroretinal.

^{15}N-Enriched purple membrane

Resonance Raman spectra of purple membrane grown in fully enriched ^{15}N media are compared in Fig. 1 to spectra of native purple membranes. In this figure spectra A and B were obtained with 457.9 nm excitation and C and D were obtained with 530.9 nm excitation to obtain scattering with (457.9 nm) and without (530.9 nm) contributions from M_{412}. In addition the results shown in Fig. 2 were obtained with a counting time per wavelength interval which was longer than the time used to obtain the results in Fig. 1. The data in Fig. 2 are displayed on an expanded scale to allow for a more careful analysis of frequency shifts in the carbon-carbon (C=C) and carbon-nitrogen (C=N and C=$\overset{\oplus}{\text{N}}$-H) stretching regions. From the data in Figs. 1 and 2 it is clear that within our ±2 cm^{-1} resolution ^{15}N enrichment affects only two vibrational modes in the region 1000 cm^{-1}—1650 cm^{-1}. These are the C=$\overset{\oplus}{\text{N}}$-H stretch which moves from 1642 cm^{-1} (^{14}N) to 1628 cm^{-1} (^{15}N) and the 1620 cm^{-1} mode (^{14}N) which moves to 1615 cm^{-1} (^{15}N). In view of these data, we feel that the 1620 cm^{-1} band is the result of a normal coordinate that includes motion of the Schiff base nitrogen and, thus, must reflect the deprotonated state of the Schiff base.

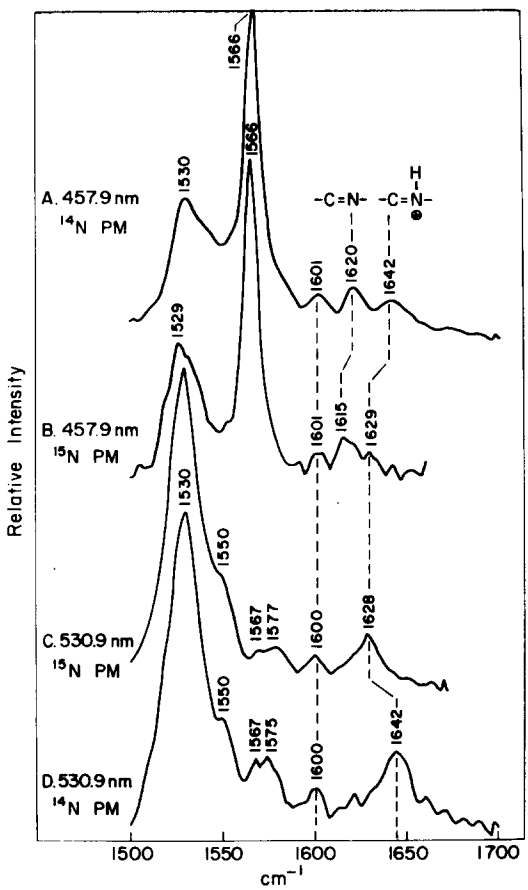


Fig. 2. Resonance Raman spectra of ^{14}N (A and D) and fully enriched ^{15}N-labelled bacteriorhodopsin (B and C), obtained with 457.9 nm (A and B) or 530.9 nm (C and D) excitation. Laser power was 50 mW, spectral resolution 2 cm^{-1}, counting time 20 s per channel. PM, purple membrane.

Therefore, this result directly confirms our earlier assingment.

This conclusion does not mean that either of the above vibrational modes are isolated carbon-nitrogen vibrations. It has already been pointed out that the C=$\overset{\oplus}{\text{N}}$-H vibrational mode in bacteriorhodopsin is 13 cm^{-1} lower in frequency than the mode in an isolated protonated retinal Schiff base in model compounds [9]. One model that effectively accounts for this observation is the suggestion that the proton on the Schiff-base is interacting with a protein residue [9]. Resuspension of native, and ^{15}N-enriched purple membrane in $^{2}H_2O$ further demonstrates the complicated nature of the carbon-nitrogen stretching modes. As can be seen in Fig. 3, resuspending ^{15}N enriched purple membrane in $^{2}H_2O$ results in only a 14 cm^{-1} shift (1628 cm^{-1} → 1614 cm^{-1}), whereas, deuterating the native ^{14}N material results in a 22 cm^{-1} shift (1642 cm^{-1} → 1620 cm^{-1}). In other words, even though ^{15}N enrichment causes a 14 cm^{-1} decrease in the C=$\overset{\oplus}{\text{N}}$-H vibrational frequency (1642 → 1628), such an enrichment results in only a 6 cm^{-1} decrease in the C=$\overset{\oplus}{\text{N}}$-^{2}H stretching frequency (see Fig. 3 and the summary of results in Fig. 4). This difference is interesting in view of the

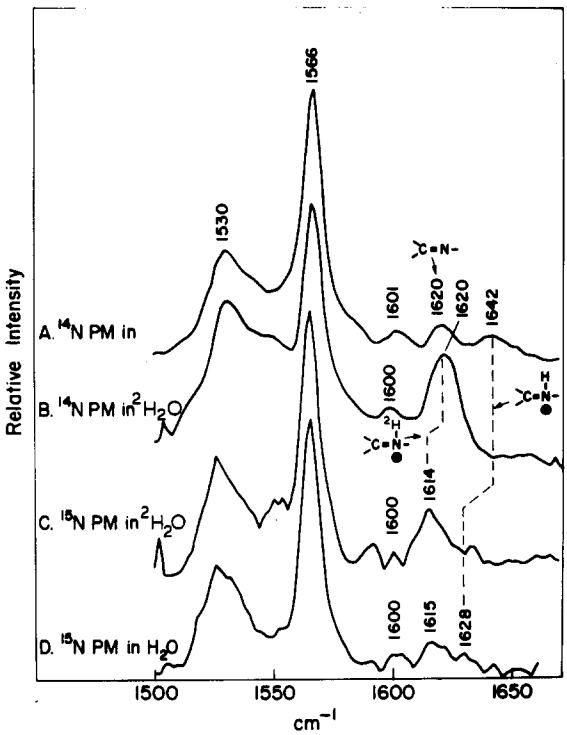


Fig. 3. Resonance Raman spectra of ^{14}N-labelled bacteriorhodopsin (A and B) and fully ^{15}N-enriched bacteriorhodopsin (C and D), suspended in H_2O (A and D) or in $^{2}H_2O$ (B and C). The spectra were excited with 50 mW of 457.9 nm light and detected with 2 cm^{-1} resolution and a counting time of 10 s per channel. PM, purple membrane.

observation that ^{15}N enrichment also shifts the 1620 cm^{-1} unprotonated Schiff base band by only 5 cm^{-1} (see Fig. 2A and B and Fig. 4). Thus, it is clear that the vibrational modes which include the protonated and unprotonated Schiff bases exhibit ^{15}N-induced frequency shifts which cannot delineate the exact composition of these normal modes. However, what does emerge from these results is the observation that the 1642 cm^{-1} and the 1620 cm^{-1} bands are the only vibrational modes that reflect the character of the Schiff-base nitrogen in its different states of protonation.

Purple membrane reconstituted with selectively deuterated [15-^{2}H]retinal

Earlier experiments had shown [8,9] that incorporating fully deuterated retinal into protonated bacterio-opsin caused a 17 cm^{-1} shift for the protonated Schiff-base mode, from 1642 cm^{-1} to 1625 cm^{-1}, whereas the corresponding effect on the unprotonated (C=N) and deuterated (C=$\overset{\oplus}{N}$-^{2}H) Schiff-base frequency was 24 cm^{-1} and 25 cm^{-1}, respectively (see Fig. 5). In order to determine which of the retinal hydrogens were causing this effect, we have obtained spectra of purple membrane incorporated with retinal containing a deuteron on the C-15 position *.

* A complete discussion and comparison of these (C_{15}-^{2}H)-labelled purple membrane spectra with the spectra of native purple membrane is described in Appendix 1. However, in this section we will focus on the effect of this selective deuteration on the Schiff-base vibrational modes.

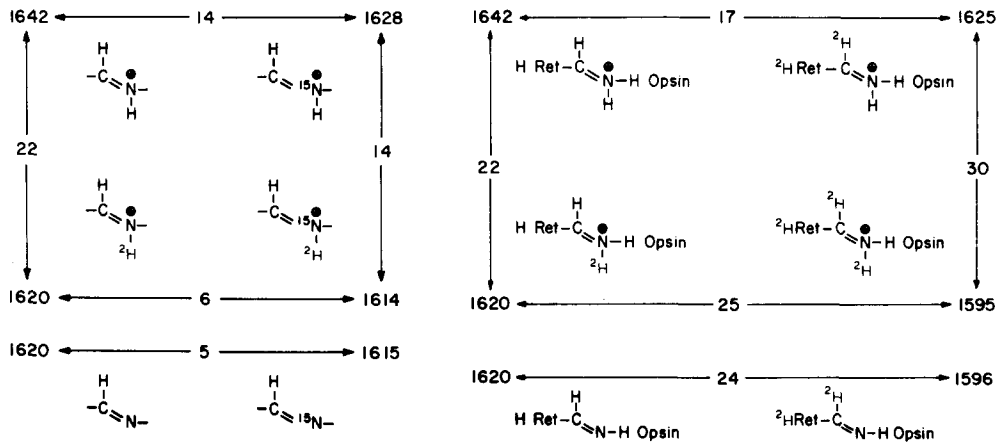


Fig. 4. Summary of the carbon-nitrogen stretching frequencies of the protonated, unprotonated and deuterated Schiff-base linkages in bacteriorhodopsin containing ^{14}N or ^{15}N.

Fig. 5. Summary of carbon-nitrogen stretching frequencies of the protonated, unprotonated and deuterated Schiff-base linkages in bacteriorhodopsin containing fully protonated or fully deuterated retinal.

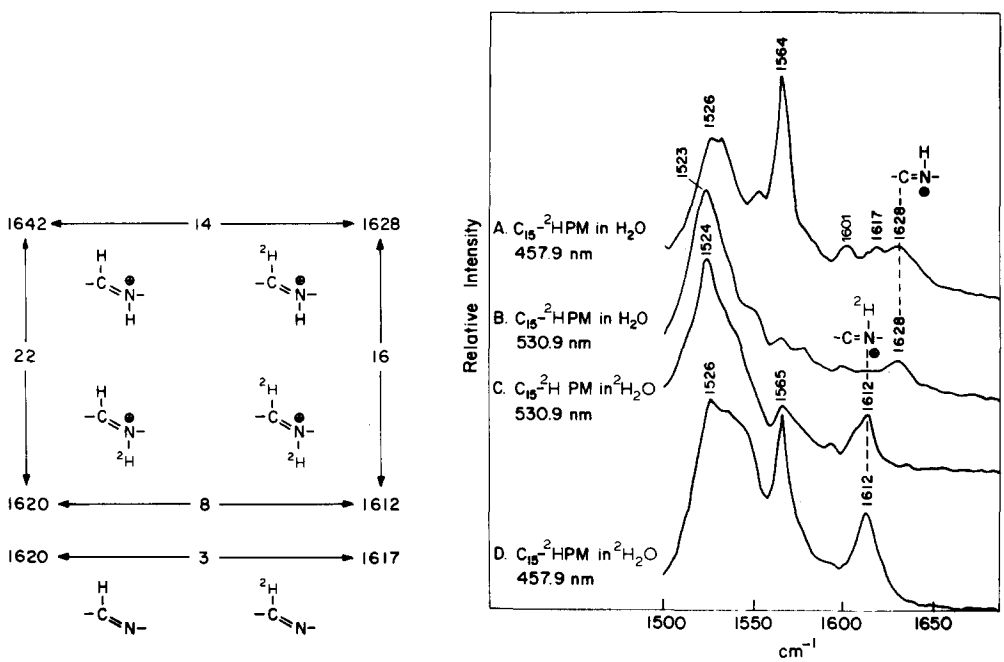


Fig. 6. Summary of carbon-nitrogen stretching frequencies of the protonated, unprotonated and deuterated Schiff-base linkages in bacteriorhodopsin containing the retinal chromophore with a proton or a deuteron at the C-15 position. The C_{15}-^{2}H C=N could be at 1617 or 1601 cm^{-1}. Further data are necessary to firmly establish this vibrational frequency. We have chosen 1617 cm^{-1} simply to be consistent with the ^{15}N shift observed for this vibrational mode.

Fig. 7. Resonance Raman spectra of bacteriorhodopsin containing retinal with a deuteron on C-15, in H_2O (A and B) and in ^2H_2O (C and D), obtained with either 457.9 nm (A and D) or 530.9 nm (B and C) excitation. Laser powers were 50 mW, spectral resolution was 2 cm^{-1} and counting time was 10 s per channel. PM, purple membrane.

These results indicate (see summary of results in Fig. 6 and compare the spectra in Figs. 7A and 2A) that deuterating C-15 causes a 14 cm^{-1} lowering of The $C=\overset{\oplus}{N}-H$ stretching frequency which is practically the entire 17 cm^{-1} shift detected when the retinal is fully deuterated (see Fig. 5). On the other hand, deuterating C-15 causes a 3 cm^{-1} (see legend to Fig. 6) and 8 cm^{-1} shift on the 1620 cm^{-1} C=N and $C=\overset{\oplus}{N}-^2H$ bands, respectively, which is, in these systems, only a fraction of the 24 cm^{-1}/25 cm^{-1} downshift observed on fully deuterating the retinal (compare Figs. 6 and 5). The simplest explanation for these results is that the full length of the isoprenoid chain, and possibly even the β-ionone ring, contribute to the C=N and the $C=\overset{\oplus}{N}-^2H$ Schiff-base modes more than they contribute to the protonated Schiff-base, $C=\overset{\oplus}{N}-H$, vibrational frequency.

To test these various alternatives for ring and chain contributions to C=N and $C=\overset{\oplus}{N}-^2H$ normal modes we have obtained spectra of bacterio-opsin reconstituted with 3-dehydroretinal. Since 3-dehydroretinal has an additional double bond in the β-ionone ring, which is conjugated to the -C=C- double bonds in the isoprenoid chain, this analog is uniquely capable of deciding whether ring and/or C=C modes are involved in the above end group vibrations.

Purple membrane reconstituted with 3-dehydroretinal

Resonance Raman spectra of this chemically modified bacteriorhodopsin show (see Fig. 8 and a detailed discussion in Appendix 2) that the C=C stretching frequencies of the BR and the M species are lowered in frequency to 1510

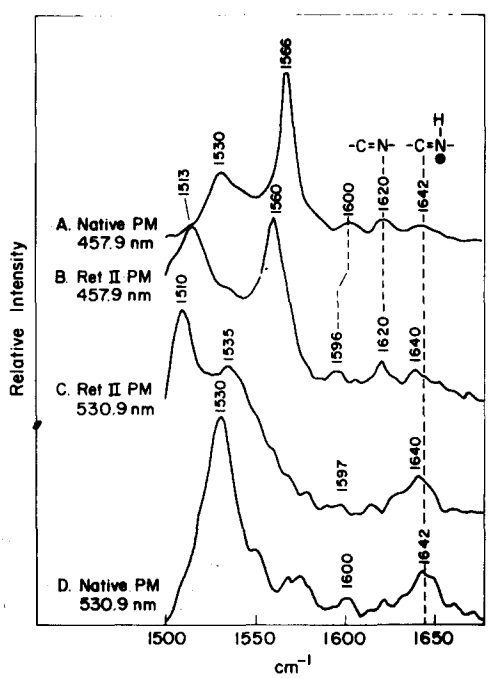


Fig. 8. Resonance Raman spectra of bacteriorhodopsin (A and D), and bacterio-opsin reconstituted with 3-dehydroretinal, retinal II (B and C), obtained with 50 mW of 457.9 nm (A and B) or 530.9 nm (C and D) excitation. PM, purple membrane.

cm^{-1} and 1560 cm^{-1}, respectively, as would be expected from the increased π electron delocalization in species which contain 3-dehydroretinal. However, the 1620 cm^{-1} unprotonated vibrational mode is completely unperturbed by this chemical modification and the protonated vibrational mode is essentially unperturbed. This is the case even though a neighboring band observed at 1601 cm^{-1} in native purple membrane spectra (a frequency region where C=C stretches could be observed) is shifted down to 1597 cm^{-1}. This indicates the lack of C=C character in the 1620 cm^{-1} band. Furthermore, these data suggest that neither the C=C nor β-ionone ring modes are involved in the 1620 cm^{-1} Schiff base vibrational mode. Therefore, to explain the 24 cm^{-1} downshift in the unprotonated Schiff base frequency on fully deuterating the retinal we are left with one possible alternative: that coupling with C-H bends on the isoprenoid chain may play an important role in the Schiff-base vibrations and, thus, could be responsible for the effects we observed.

Coupling of C-H bends has been invoked in previous attempts to understand unconventional deuterium isotope effects on the resonance Raman spectra of bacteriorhodopsin [8]. For example, it has been observed that a new band appears in the C-H bend region upon replacement of the Schiff base proton with a deuteron. This band is at higher frequency than the 960 cm^{-1} band observed in native purple membrane which is in the wrong direction for a simple mass effect [8,11]. Marcus and Lewis [8] have argued, based on other isotope effects and on the results of Craig and Overend [20] on simpler molecular systems, that N-^{2}H/C-H coupling could be responsible for such an upshift. Such coupling may also account for the altered isotopic shifts in the C=N and $C=\overset{\oplus}{N}$-^{2}H systems upon ^{15}N enrichment, C-15 selective deuteration and full deuteration of the retinal.

In conclusion, using ^{15}N enrichment we have unequivocally assigned the vibrational modes which reflect the protonated and unprotonated character of the Schiff-base linkage. In addition, we have demonstrated that the C_{15}-H bond contributes a smaller fraction to the C_{15}=N and $C_{15}=\overset{\oplus}{N}$-^{2}H vibrational modes than to the $C_{15}=\overset{\oplus}{N}$-H band. Conversely, the C-H bonds on the rest of the isoprenoid chain participate to a greater extent in the C_{15}=N and $C_{15}=\overset{\oplus}{N}$-^{2}H vibrational modes than in the $C_{15}=\overset{\oplus}{N}$-H band. Furthermore, using purple membranes reconstituted with 3-dehydroretinal, we have shown that ring modes or C=C stretches do not, in all probability, contribute to either one of the 1620 cm^{-1} unprotonated vibrational modes. Based on these data we will reexamine in the following paper the kinetics of Schiff-base deprotonation using kinetic resonance Raman spectra of isotopically labelled purple membranes in various enviroments.

Appendix 1

In this appendix the spectra of purple membrane selectively enriched with a deuteron at the C-15 position is compared to naturally occuring fully protonated purple membranes.

In addition to the changes already discussed in the text of the paper, several other interesting alterations can be seen in the 530.9 nm spectra of the (C_{15}-^{2}H)-labelled membranes. There is a downshift (see Fig. 9 C and D) in the C=C vibra-

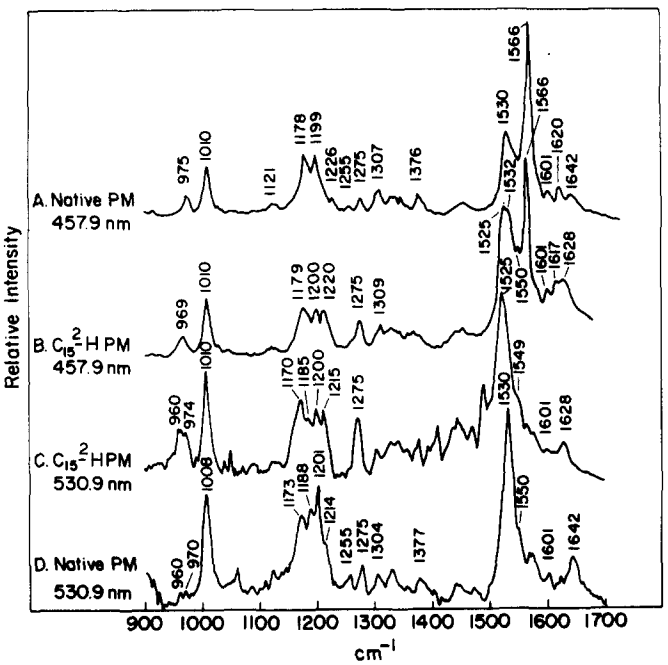


Fig. 9. Resonance Raman spectra of bacteriorhodopsin (A and D) and of bacterio-opsin reconstituted with retinal deuterated at C-15 (B and C), obtained with 50 mW of either 457.9 nm (A and B) or 530.9 nm (C and D) excitation. Spectral resolution was 2 cm^{-1} and counting time was 10 s per channel. PM, purple membrane.

tional mode from 1530 cm^{-1} to 1525 cm^{-1}. There is considerably greater strength relative to the mode at ~1200 cm^{-1} in the modes around 1275 cm^{-1}, 1215 cm^{-1}, 1185 cm^{-1} and 1170 cm^{-1}; Furthermore, there are two additional alterations in modes which previous results indicated [21] may involve the end group. These are a band at 1255 cm^{-1} in normal membranes which is absent in the (C_{15}-^{2}H)-labelled sample, and bands between 950 and 990 cm^{-1}, which appear to be significantly effected by this modification at least in terms of their intensity.

The photostationary results at 457.9 nm (see Fig. 9A and B) are not as clear cut in the analysis of changes in the normal modes, since several additional species in the photostationary mixture are resonantly enhanced with this laser frequency. However, certain interesting changes are observed and these will now be discussed. Firstly, even though the BR_{570} 1530 cm^{-1} C=C stretching mode has been shifted to 1525 cm^{-1} (see above discussion of 530.9 nm spectra and compare 457.9 nm spectra, Fig. 9A and B) the M_{412} C=C stretch is unshifted by this substitution. Secondly, an observation of considerable potential interest is the fact that the intensity of the 1566 cm^{-1} C=C stretch of M_{412}, when compared to the intensity of the BR_{570} 1530 cm^{-1} C=C stretch is observably decreased upon C_{15}-^{2}H substitution (compare Fig. 9A and B). Furthermore, another example of this alteration in the steady state composition are the clearly observable bands at 1550 cm^{-1} and 1532 cm^{-1}. The 1550 cm^{-1} band has been assigned previously to an intermediate X [8] and appears to be unchanged

in frequency in the C_{15}-^{2}H spectra. On the other hand, the 1532 cm^{-1} vibrational mode in the C_{15}-^{2}H spectra may be correlated with the 1537 cm^{-1} band we have observed previously [8]. This band has been associated in kinetic spectra with the protonated L intermediate. However, in the stationary state experiments reported here, it could also be associated with the later intermediate N. Thus, it appears that C_{15}-^{2}H substitution does not effect the C=C stretch of the unprotonated intermediates M and X, but does alter the 1530 cm^{-1} BR_{570} and 1537 cm^{-1} (L_{550} or N_{520}) vibrations of the protonated species. Finally, the effect of this C_{15}-^{2}H substitution on the modulation of photostationary intermediate kinetics may help elucidate isomerizations of the chromophore in the evolution of M_{412} and deserve further investigation.

Appendix 2

In this appendix the resonance Raman spectra of 3-dehydrobacteriorhodopsin will be discussed over the frequency range 900—1700 cm^{-1}. Previously in this paper we have considered the frequency range 1500—1700 cm^{-1} and noted that the C=N vibrational frequency is unchanged by replacement of the retinal in purple membrane with 3-dehydroretinal. However, such a modification of the system does result in considerable changes in the rest of the vibrational spectrum.

The intense band in the 530.9 nm spectrum of 3-dehydrobacteriorhodopsin (see Fig. 10A) is the 1510 cm^{-1} C=C stretching mode which normally occurs at 1530 cm^{-1} in purple membrane. This lowered frequency is understandable in terms of increased electron delocalization which also causes the red shift in the absorption maximum of 3-dehydrobacteriorhodopsin (600 nm) compared to natively occurring purple membrane, absorbing maximally at 570 nm. This absorption red shift may bring into resonance intermediates that normally would not contribute to the resonance Raman spectra obtained with 530.9 nm excitation. Such an effect may explain the origin of the strong band at 1535 cm^{-1}. The lack of any unprotonated C=N stretch at 1620 cm^{-1} in the 530.9 nm spectrum indicates that the 1536 cm^{-1} band cannot arise from either M or X intermediates but may be associated with L or N. Yet, it is hard to see how L could be responsible for this band because in native purple membrane, C=C stretch of L is observed at the same frequency [8] and, thus, this would require that 3-dehydroretinal substitution would not effect the C=C stretch of L while it does effect the C=C stretch of all the other observed species. Such an argument may associate this band with N. An alternate explanation arises from the observation [21] that 3-dehydroretinal normally exhibits multiple C=C stretching modes and, therefore, the 1536 cm^{-1} band could also arise from the original BR species. However, it should be noted in this regard that in 3-dehydroretinal the multiple C=C stretching modes are separated by approximately half the magnitude we observe in the 530.9 nm spectrum.

In the rest of the 530.9 nm spectrum a band with greater intensity appears at 1320 cm^{-1} in the 3-dehydro system. On the other hand, bands at 1278 cm^{-1} and 1256 cm^{-1} are similar in intensity and frequency in both membranes. The band intensities in the fingerprint region are considerably altered in the 3-dehydro membranes but the frequencies are close to those observed in native

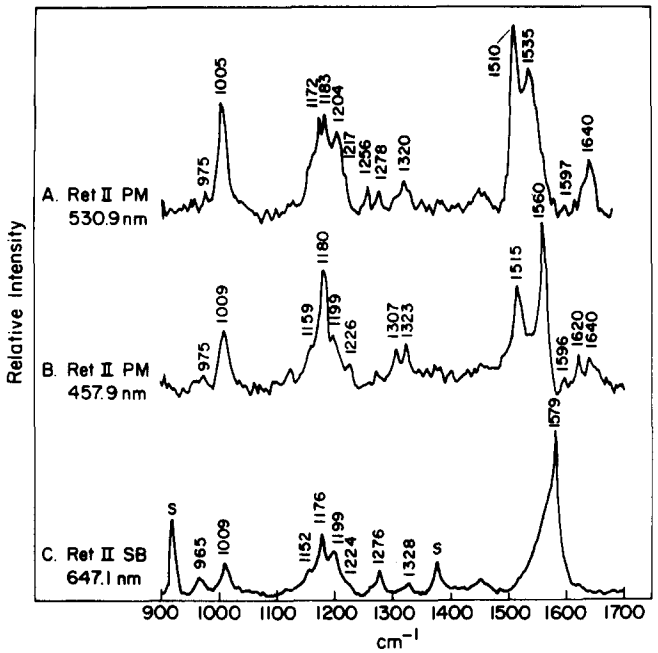


Fig. 10. Resonance Raman spectra of bacteriorhodopsin reconstituted with 3-dehydroretinal, retinal II, excited with 50 mW of 530.9 nm light (A), or with 50 mW of 457.9 nm light (B), and the Raman spectrum of the unprotonated Schiff base of retinal II with butylamine in CH_3CN excited with 10 mW of 647.1 nm (C), S indicates solvent Raman bands. PM, purple membrane.

membranes. It is not yet clear whether these intensity changes arise from intrinsic differences in the BR spectrum or from alterations due to contributions from additional intermediates. However, it should be noted that the intensity changes observed in going from the 530.9 nm spectrum of native membranes to the 530.9 nm 3-dehydro spectrum are quite similar to intensity changes observed between all-*trans*-retinal and all-*trans*-3-dehydroretinal [21].

The spectrum of 3-dehydrobacteriorhodopsin excited with 457.9 nm (Fig. 10B) shows an intense peak in the C=C stretching region at 1560 cm^{-1} which is apparently due to enhancement of the 3-dehydro M intermediate. Concomitantly a band at 1620 cm^{-1} is observed which arises from the unprotonated C=N stretch. The M C=C stretch is reduced in frequency by 6 cm^{-1} when compared to the C=C stretch of M_{412}. In addition, there is a band at 1515 cm^{-1} which appears to be associated with residual resonance enhancement at 457.9 nm of the BR species. However, if indeed this C=C stretch is associated with the BR species and not with an additional M C=C stretch, then there is a frequency shift in this band on going from 530.9 nm excitation to 457.9 nm excitation (see Fig. 10 A and B). A similar frequency shift is noted in the C-CH_3 stretch which moves from 1004 cm^{-1} (at 530.9 nm) to 1010 cm^{-1} (at 457.9 nm), whereas, in native membrane such a shift is not observed. These shifts could be associated with either changes in steady state intermediate concentrations and/or excited state effects which depend on excitation frequency. It is interesting to note that in native purple membrane we observe a 3 cm^{-1}

decrease in the C=C stretching frequency of BR_{570} in going from 457.9 nm excitation to 568.2 nm excitation (see Fig. 1 of the following paper [22]).

The fingerprint region of the 3-dehydro system is vastly different when spectra obtained with 457.9 nm and 530.9 nm excitation are compared. The 1183 cm^{-1} band in the 530.9 nm spectrum moves to 1180 cm^{-1} and becomes the dominant feature in this region. In addition, the 1204 cm^{-1} band decreases significantly in intensity and moves to 1199 cm^{-1}. Furthermore, the strong 1172 cm^{-1} band and the weak 1256 cm^{-1} band in the 530.9 nm spectrum have disappeared and a new band characteristic of unprotonated Schiff bases is detected at 1226 cm^{-1}. There also appears to be a weak band at 1159 cm^{-1}. Changes in intensity are also noted in the 1300—1350 cm^{-1} region. These drastic spectral alterations probably arise from the fact that the M species of the 3-dehydro pigment is resonance enhanced to a greater extent by 457.9 nm excitation than the M_{412} species of native membranes. Finally, it is interesting to note that the 457.9 nm 3-dehydro spectrum is quite similar in the fingerprint region to the spectrum of an unprotonated Schiff base of *trans*-3-dehydroretinal (see Fig. 10C).

Acknowledgements

This work was supported by a National Institutes of Health Grant No. EY01377 and the Division of Basic Energy Sciences of the Department of Energy.

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