Rhodopsin–Lumirhodopsin Phototransition of Bovine Rhodopsin Investigated by Fourier Transform Infrared Difference Spectroscopy†

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ABSTRACT: The rhodopsin–lumirhodopsin transition has been investigated by Fourier transform infrared difference spectroscopy using isotope-labeled retinals. In the transition, two protonated carboxyl groups are involved. Another carbonyl band, located at 1725 cm^{-1} in rhodopsin, is shifted to 1731.5 cm^{-1} in lumirhodopsin. This line is tentatively assigned to a carbonyl stretching vibration of a peptide bond adjacent to the nitrogen of a proline residue. The C=N stretching vibration of rhodopsin could unequivocally be assigned to a band at 1659 cm^{-1}. In contrast to rhodopsin and bathorhodopsin, the C=N stretching vibration of lumirhodopsin is at a low position, i.e., at 1635 cm^{-1}, and exhibits only a downshift of 4 cm^{-1} upon deuteriation of the nitrogen. The C_{15}–H rocking vibration of rhodopsin is assigned to the unusual high position of 1456 cm^{-1} and shifts into the normal region upon formation of lumirhodopsin. From these results, it is concluded that, whereas the environment of the Schiff base in rhodopsin, bathorhodopsin, and isorhodopsin is approximately the same, large changes occur with the formation of lumirhodopsin. From the assignment of the C_{10}–C_{11} stretching vibration in bathorhodopsin and lumirhodopsin, a 10-s-cis geometry of lumirhodopsin can be excluded.

The process of vision is initiated by the absorption of light by rhodopsin. Rhodopsin is an 11-*cis*-retinal-containing protein located in the disk membrane of the rod outer segment of the photoreceptor cell. After the absorption of light, rhodopsin decays through a series of intermediates (bathorhodopsin, lumirhodopsin, meta I, and meta II) to *all-trans*-retinal and the protein opsin. The meta II intermediate then initiates, by a sequence of enzymic reactions, a change in the electrical properties of the cell and thus the neural signal.

Whereas there is general agreement that the photoreaction involves isomerization of the retinal from 11-cis to all-trans, different views are still held on the structure of bathorhodopsin. Bagley et al. (1985), using Fourier transform infrared spectroscopy (FTIR)[1] and Palings et al. (1987), using resonance Raman spectroscopy, suggested a distorted all-trans configuration. Liu and Asato (1985) proposed a 10-s-cis configuration which thermally converts to the all-trans conformer in the lumirhodopsin intermediate. This suggestion was strongly challenged by the results of Sheves et al. (1986), who found identical decay kinetics of the bathorhodopsin–lumirhodopsin transition in normal rhodopsin and rhodopsin regenerated with a modified retinal containing a 10,11-s-trans bond fixed by a six-membered ring. The problem of the geometry of the 10,11-s bond was especially addressed by Palings et al. (1987) in a detailed resonance Raman study using isotope-labeled retinals. In a normal polyene, an s-cis bond would result in a downshift of the corresponding C–C stretching vibration of 100 cm^{-1} (Smith et al., 1986). From its downshift upon labeling with ^{13}C at C_{10} and C_{11}, Palings et al. (1987) assigned the band at 1166 cm^{-1} to the C_{10}–C_{11} stretching vibration and concluded that this high position is compatible only with a 10-s-trans geometry. Earlier, Bagley et al. (1985), using similar arguments but with more indirect experimental evidence, arrived at the same conclusions. However, for protonated retinylidene Schiff bases, these arguments were questioned on the basis of theoretical considerations (Tavan & Schulten, 1986). They have shown that the geometrical effect could be compensated for by the π-electron system. Thus, especially if the delocalization of the electronic system is large, the position of the C–C stretching band may not in all cases allow an unequivocal conclusion on the corresponding geometry.

Earlier, difference spectra of the rhodopsin–bathorhodopsin, rhodopsin–isorhodopsin, and rhodopsin–meta II transitions were reported (Siebert et al., 1983; Rothschild et al., 1983). An investigation of the primary photochemistry, using labeled retinals, was performed by Bagley et al. (1985). From these investigations and from the resonance Raman studies performed by Palings et al. (1987), it appears that in bathorhodopsin the retinal had an electronically and geometrically distorted structure. To deduce the geometry of the retinal in the protein, an internal reference is therefore needed. It is generally accepted that in lumirhodopsin the geometry is all-trans. Since the absorption maximum is 5 nm downshifted as compared to rhodopsin, the electronic distortion observed in bathorhodopsin should be largely relaxed. An assignment of the retinal modes, especially of the C_{10}–C_{11} stretching vibration, in lumirhodopsin together with that of rhodopsin and bathorhodopsin (Palings et al., 1987; Bagley et al., 1985) should therefore serve as a reference for the deduction of the retinal geometry in bathorhodopsin.

The meta II state seems to play an essential role in the transduction process. It activates the enzymatic cascade by triggering the GDP–GTP exchange of the G protein. The Schiff base is deprotonated, and it has been shown recently

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[1] Abbreviations: DTT, dithiothreitol; FTIR, Fourier transform infrared spectroscopy; GDP, guanidine diphosphate; GTP, guanidine triphosphate; HOOP, hydrogen out of plane vibration; ROS, rod outer semgnent(s); RR, resonance Raman spectroscopy.

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that the deprotonation is a prerequisite for the activation of the G protein (Longstaff et al., 1986). Therefore, the molecular causes leading to the deprotonation of the Schiff base are of special interest. In a recent publication, we have shown that in bacteriorhodopsin an isomerization of the retinal from all-trans to 13-cis, 14-s-cis has taken place (Gerwert & Siebert, 1986). The distorted 14-s-cis geometry may be an essential cause for the deprotonation of the Schiff base in M_{412}. For rhodopsin, however, such a mechanism can be excluded, since in meta I the chromophore is all-trans. Therefore, a different mechanism for the deprotonation of the Schiff base, probably based on changes in the environment, has to be assumed. Such changes in the environment can be detected by monitoring the vibrations of this group, i.e., the C=N stretching and the N–H and the C_{15}–H bending vibrations. IR and RR investigations have already shown that in bathorhodopsin the Schiff base is not altered appreciably (Siebert et al., 1983; Bagley et al., 1985; Palings et al., 1987). Therefore, to explain the deprotonation at meta II, the surrounding of the Schiff base must change in the later intermediates. Earlier, it was shown that the rhodopsin–meta II difference spectra are characterized by large molecular changes of the protein (Siebert et al., 1983; Rothschild et al., 1987). In contrast, only minor changes are observed for the rhodospin–bathorhodopsin transition. Therefore, it is of interest whether or not these changes already occur at earlier intermediates.

All these reasons prompted us to investigatge the rhodopsin–lumirhodopsin transition in greater detail. By using rhodopsin regenerated with isotopically labeled retinals (10,11-^{13}C, 14-^{13}C, 15-^{13}C, 14,15-^{13}C, C_{14}-^{2}H, C_{15}-^{2}H, and C_{19}-$^{2}H_3$), we assigned most of the vibrations of the terminal end of the chromophore. A basis for the assignments was given by the vibrational analysis of retinal protonated Schiff base model compounds by Smith et al. (1985).

Materials and Methods

All manipulations involving rhodopsin were performed under dim red light or in darkness. ROS were isolated as previously reported (Siebert et al., 1983) and stored at 200 K. The [C_{15}-^{2}H]retinal was obtained by reducing retinoic acid with $LiAl^{2}H_4$ in dry ether to [C_{15}-$^{2}H_2$]retinol and subsequent oxidation of the retinol to [C_{15}-^{2}H]retinal with MnO_2. The [C_{14}-^{2}H]retinal was synthesized similarly as described by Hagemeier (1985). The other isotope-labeled retinals were a generous gift from J. Lugtenburg. All labeled retinals were photochemically isomerized, and the 11-*cis*-retinal was purified by HPLC. For regeneration, rhodopsin was bleached in a solution of 0.1 M phosphate buffer, pH 7, 0.01 M hydroxylamine, and 1 mM DTT at 293 K with white light. To remove the hydroxylamine, the bleached membranes were washed 3 times with a solution of 0.01 M phosphate buffer, pH 6, and 1 mM DTT and resuspended in a solution of 1 mM phosphate buffer, pH 6, and 1 mM DTT. For regeneration, a 3-fold molar excess of the labeled retinal dissolved in ethanol was added, and the regeneration mixture was gently stirred for 12 h.

For the infrared investigations, approximately 5 nM rhodopsin of the ROS suspension was dried under a stream of dry nitrogen onto a AgCl window and rehydrated with H_2O or $^{2}H_2O$. The films were sealed with a germanium window and mounted in a continuous-flow cryostat equipped with CsJ windows. The samples were cooled to the desired temperature (77 K for the bathorhodopsin and isorhodopsin, 173 K for lumirhodopsin, and 240 K for the meta I intermediate). To produce the bathorhodopsin, lumirhodopsin, and meta I intermediates, the samples were illuminated with light of wavelengths between 435 and 470 nm for 4 mins. To obtain isorhodopsin, they were illuminated for 20 min with light of wavelengths longer than 570 nm.

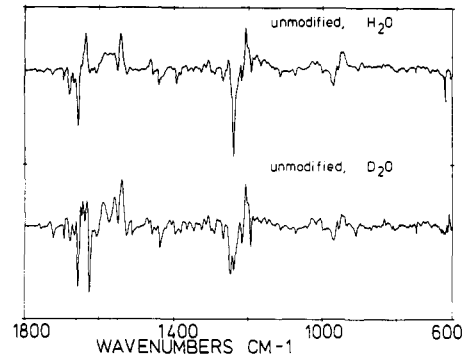

FIGURE 1: Rhodopsin–lumirhodopsin difference spectra for H_2O and $^{2}H_2O$.

Spectral analysis in the visible range showed that less than 15% isorhodopsin is present in the photoproduct. Since it is difficult to deduce more precise numbers from retinal extraction and subsequent HPLC analysis, another method was employed. From the rhodopsin–lumirhodopsin difference spectrum, various fractions of the rhodopsin–isorhodopsin difference spectrum were subtracted. To determine the subtraction constant, several characteristic isorhodopsin bands were used at positions where no rhodopsin bands showed up in the rhodopsin–bathorhodopsin difference spectra. In this way, it could be deduced that less than 5% of the photolyzed rhodopsin was transformed to isorhodopsin. The characteristic bands chosen are at 1691, 1343, 1166, and 1013 cm^{-1} in H_2O and at 1341 cm^{-1} in $^{2}H_2O$ (Siebert et al., 1983). This small amount of isorhodopsin does not influence the difference spectra.

The IR difference spectra were obtained on a Bruker FT-IR Model IFS 113v spectrophotometer equipped with a HgCdTe detector. The measurements were carried out as previously reported (Siebert et al., 1983). First, a single-beam spectrum of the unilluminated sample was recorded. The sample was then illuminated, and a second single-beam spectrum was recorded, and from the single-beam spectra, the rhodopsin–photoproduct difference spectrum was calculated. For each single-beam spectrum, 1024 scans were accumulated. They were divided in 4 blocks of 256 scans each for base-line control as described in Siebert et al. (1983). To increase the signal/noise ratio, the difference spectra of different samples were co-added.

To make the interpretation of the difference spectra easier, the spectrum of the unmodified rhodopsin was subtracted from that of the isotope-labeled ones. The constants for the subtractions were obtained by linear regression of the distances between the subtracted spectra in the range of 1800–800 cm^{-1} using the software of the Bruker FTIR instrument (Gerwert & Siebert, 1986). In the subtraction, only bands are present which are sensitive to isotopic labeling. Positive bands are caused by unshifted rhodopsin or shifted lumirhodopsin lines; negative bands are due to shifted rhodopsin or unshifted lumirhodopsin vibrations. For spectral regions that are insensitive to the isotopic label, a flat line results. This feature served as a control for the reliability of the subtraction.

Results

In Figure 1, the rhodopsin–lumirhodopsin difference spectra for H_2O and $^{2}H_2O$ are shown. Positive lines are caused by lumirhodopsin and negative lines by rhodopsin. In the spectral region between 1800 and 1700 cm^{-1}, only carbonyl stretching

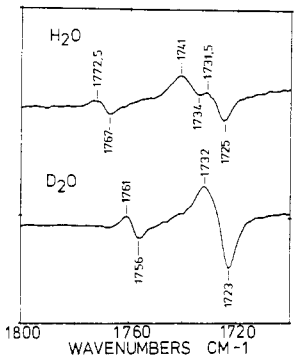

FIGURE 2: Rhodopsin–lumirhodopsin difference spectra in the region of the carboxyl C=O stretching frequency for H_2O and ^2H_2O.

vibrations of carboxylic acids and esters are expected. The enlarged spectra of this region are shown in Figure 2. At 1772.5 cm^{-1}/1767 cm^{-1}, a difference band is observed in H_2O which is shifted to 1761 cm^{-1}/1756 cm^{-1} in ^2H_2O. At 1741 and 1731.5 cm^{-1}, two lumirhodopsin lines appear, and at 1734 and 1725 cm^{-1}, two rhodopsin lines appear. The lines at 1734 and 1731.5 cm^{-1} are quite weak. A possible explanation is that they overlap each other in the difference spectrum. On deuteriation with ^2H_2O, the peaks at 1741 and 1734 cm^{-1} disappear; the peak at 1725 cm^{-1} is downshifted by 2 cm^{-1} and gains intensity as does also the peak at 1731.5 cm^{-1}. The simplest interpretation for these bands is two carbonyl groups of which the environment is altered during the rhodopsin–lumirhodopsin transition. Whereas the group which causes the lines at 1741 cm^{-1}/1734 cm^{-1} is sensitive to H/^{2}H exchange as shown by its downshift of 12 wavenumbers, the carbonyl group at 1725 cm^{-1}/1731.5 cm^{-1} does not exhibit a corresponding shift. To enhance the H/^{2}H exchange reaction, rhodopsin was bleached and regenerated in ^2H_2O, but even the exchange in the more open meta II conformation and in the apoprotein did not evoke a shift of this band.

In the region between 1700 and 1600 cm^{-1}, the C=N stretching vibration of the Schiff base and some of the C=C stretching vibrations are the only bands expected for the retinal chromophore. Figure 1 shows many more lines in this spectral region, which are probably due to alterations in the protein moiety. Because of the complexity of the spectrum, it becomes difficult to detect the Schiff base C=N stretches. However, by subtraction of the spectrum of the unmodified rhodopsin from the spectrum of the 15-^{13}C-labeled one, we can easily reveal these vibrations. In Figure 3A, the difference spectrum of the 15-^{13}C-labeled rhodopsin and the subtraction are shown. In the subtraction, the number of peaks in this spectral region is greatly reduced. The band at 1659 cm^{-1} is the C=N stretch of the unmodified rhodopsin which is shifted to 1636.5 cm^{-1} on 15-^{13}C labeling. The 1636.5 cm^{-1} peak is also caused by the unmodified lumirhodopsin C=N stretch which is shifted to 1624 cm^{-1}. In the spectrum of unmodified rhodopsin, a positive line appears at 1635.5 cm^{-1}. Thus, we assign this line to the C=N stretch lumirhodopsin. Since the 15-^{13}C-shifted rhodopsin mode absorbs at 1639 cm^{-1}, these two bands co-add to the 1636.5 cm^{-1} line in the subtraction. In Figure 3B, the corresponding spectra of the pigments in ^2H_2O are shown. For 15-^{13}C, three lines are observed above 1600 cm^{-1} in the subtraction. The negative line at 1603 cm^{-1} is also apparent in the rhodopsin–bathorhodopsin difference spectra (Bagley et al., 1985; our own unpublished results) and is assigned to the ^{13}C=N^{2}H vibration of rhodopsin. The vibration of the C=N^{2}H group is assigned to 1623 cm^{-1} in rhodopsin. The remaining negative band at 1631.5 cm^{-1} is caused by the unshifted C=N^{2}H stretch of lumirhodopsin due to its shift on

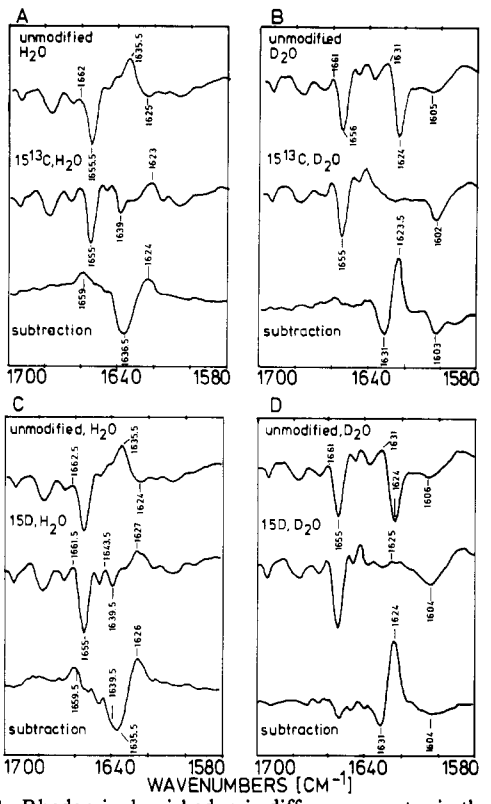

FIGURE 3: Rhodopsin–lumirhodopsin difference spectra in the region of the Schiff base stretching vibration. (Upper trace) Unmodified rhodopsin; (middle trace) labeled rhodopsin; (lower trace) subtraction of the unmodified spectrum from the modified one. (A) 15-^{13}C, H_2O; (B) 15-^{13}C, ^2H_2O; (C) C_{15}-^{2}H, H_2O; (D) C_{15}-^{2}H, ^2H_2O.

Table I: Schiff Base Frequencies As Assigned for Rhodopsin and Its Photoprodocts[a]

	rhodopsin	iso	batho	lumi	meta I
HC=NH	1659	1659*	1659*	1635	1652
HC=N^{2}H	1623	1631	1623	1631	1630
^{2}HC=NH	1639	1639*	1639*	1627	1635
C_{15}-^{2}H	1010	1000	1006	933	
C_{15}H	1456	1456*	1456*		

[a] Positions marked with an asterisk have been deduced from the absence of the rhodopsin band in the respective difference spectra.

15-^{13}C labeling. The ^{13}C-shifted C=N^{2}H vibration of lumirhodopsin probably absorbs around 1610 cm^{-1} with low intensity. Figure 3C shows the difference spectra of unmodified rhodopsin and its C_{15}-^{2}H derivative and their subtraction. Deuteriation at C_{15} shifts the Schiff base stretch of rhodopsin to 1639.5 cm^{-1} and that of lumirhodopsin to 1627 cm^{-1}. The ^{2}CH=N^{2}H mode of rhodopsin can be assigned to the broad absorption at 1604 cm^{-1} (Figure 3D). Because the 1624.5 cm^{-1} line in the subtracted spectra of Figure 3D has at least double the intensity of the 1631.5 cm^{-1} line, we assign the the 1624.5 cm^{-1} line to the HC=N^{2}H stretching mode of rhodopsin and the ^{2}HC=N^{2}H stretching mode of lumirhodopsin (see Table I for a summary of the C=N modes).

The ethylene modes of the retinal absorb in the region between 1620 and 1500 cm^{-1}. The rhodopsin–lumirhodopsin difference spectra in this frequency range of unlabeled and labeled rhodopsin are shown in Figure 4. The rhodopsin and the lumirhodopsin lines overlap each other in the difference spectra so that the intensity of the ethylene modes as compared with that of the bacteriorhodopsin system is low. In addition, mainly lumirhodopsin bands appear in the spectra: Three lines with maxima at 1584, 1569, and 1558.5 cm^{-1} contribute to a broad absorption between 1590 and 1555 cm^{-1}; a stronger

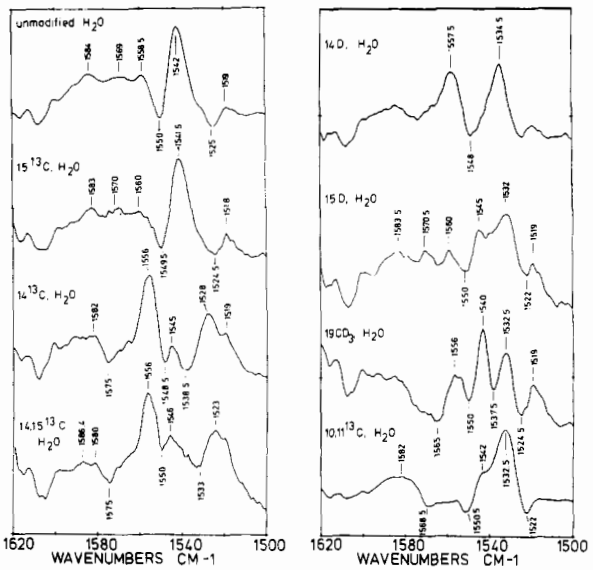

FIGURE 4: Rhodopsin–lumirhodopsin difference spectra of the spectral region of the ethylene mode for H_2O for various isotopic labels.

line is located at 1542 cm^{-1}. Rhodopsin lines appear at 1550 and 1525 cm^{-1} (Figure 4).

As expected, the rhodopsin–lumirhodopsin difference spectrum is not altered in rhodopsin regenerated with [15-^{13}C]retinal. On ^{13}C labeling at the C_{14}-position, the band at 1569 cm^{-1} disappears. Most of the intensity of the strong 1542 cm^{-1} line disappears, and two lines appear at 1528 and 1556 cm^{-1}. A small band remains at 1545 cm^{-1}. The 1550 cm^{-1} band of rhodopsin is shifted to 1548.5 cm^{-1}, and a new band appears at 1538.5 cm^{-1}. Again, only minor changes occur on additional labeling at C_{15}. Deuteriation at C_{14} also produces two lumirhodopsin lines, at 1557.5 and 1534.5 cm^{-1}. In the spectrum of the C_{15}-^2H derivative, the 1542 cm^{-1} line is split in two lines at 1545 and 1532 cm^{-1}. In the spectra of rhodopsin regenerated with [10,11-^{13}C]retinal, a broad lumirhodopsin line centered at 1582 cm^{-1} appears, and the 1542 cm^{-1} line is shifted to 1532.5 cm^{-1} with a shoulder remaining at 1542 cm^{-1}. A new rhodopsin line appears at 1568.5 cm^{-1}. In the spectrum of C_{19}-$^2H_3$-labeled rhodopsin, the 1569 cm^{-1} lumirhodopsin band disappears; instead, a rhodopsin line appears at 1565 cm^{-1}. The 1542 cm^{-1} lumirhodopsin line is split into two lines at 1540 and 1532 cm^{-1}.

In the spectral region between 1500 and 1280 cm^{-1} (Figure 5), the deformation vibrations of the methyl groups and the olefinic hydrogen in-plane rocks of the retinal are located. Again, there are many more lines in the difference spectrum of Figure 5 than one would expect for the protonated retinal Schiff base chromophore. The frequency of the C_{15}–H and the N–H bending modes is important for the molecular interpretation of the C=N stretch. Deuteriation shifts the rocks to the spectral region below 1000 cm^{-1}. Deuteriation of the medium causes many small changes in the spectrum. At 1471 cm^{-1}, a new lumirhodopsin line appears, and at 1454 cm^{-1}, a small rhodopsin line shifts up to 1458 cm^{-1} (Figure 5A,B, top). At 1390.5 cm^{-1}, a rhodopsin line disappears, a new line appears at 1394.5 cm^{-1}, and a new positive line appears at 1400 cm^{-1}. The same behavior is also apparent in the rhodopsin–bathorhodopsin difference spectra (Siebert et al., 1983). A new line appears in the spectrum for deuteriated rhodopsin at 907 cm^{-1} (Figure 6) which may represent the N–^2H bending mode. In the spectrum of the C_{15}–^2H derivative in ^2H_2O, this band is located at 909 cm^{-1} (data not shown).

In the spectra obtained with opsin regenerated with [C_{15}-^2H]retinal (Figure 5A,B), a rhodopsin line at 1454 cm^{-1}

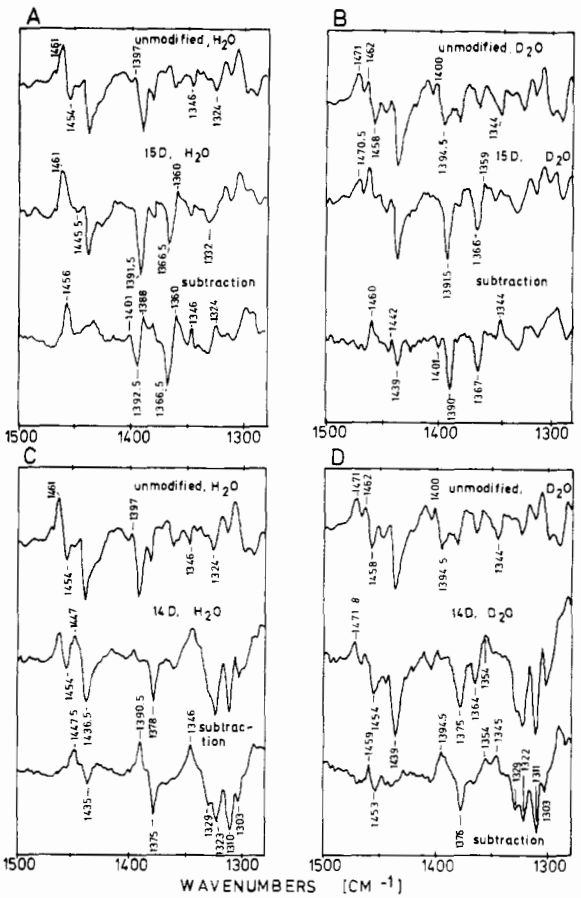

FIGURE 5: Rhodopsin–lumirhodopsin difference spectra of the C–H bending modes. (Upper trace) Unmodified rhosopsin; (middle trace) labeled rhodopsin; (lower trace) subtraction of the unmodified spectrum from the modified one. (A) C_{15}-^2H, H_2O; (B) C_{15}-^2H, ^2H_2O; (C) C_{14}-^2H, H_2O; (D) C_{14}-^2H, ^2H_2O.

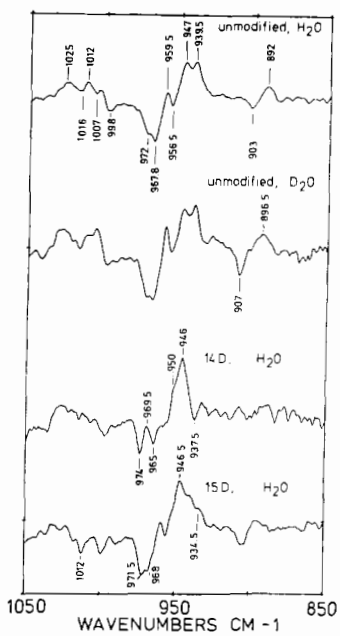

FIGURE 6: Rhodopsin–lumirhodopsin difference spectra below 1000 cm^{-1} for various isotopic labels.

disappears (1458 cm^{-1} in ^2H_2O), and a new line appears at 1012.5 cm^{-} (Figure 6). In the subtraction, the disappearing line is at 1456 cm^{-1} in H_2O (1460 cm^{-1} in ^2H_2O). Thus, we assign the 1456 cm^{-1} line to the C_{15}–H bending mode of rhodopsin and the corresponding C_{15}–^2H mode to 1012.5 cm^{-1}. The lumirhodopsin C_{15}–^2H bending mode absorbs at 934.5

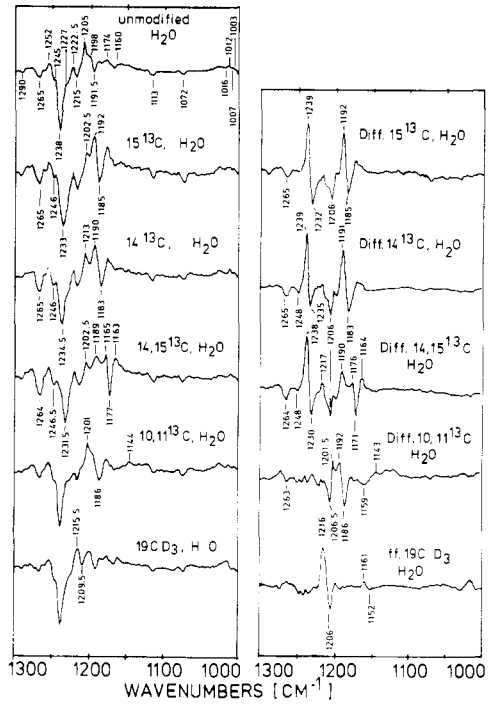

FIGURE 7: Rhodopsin–lumirhodopsin difference spectra in the region of the C–C stretching vibrations measured in H_2O. (Left traces) Unmodified rhodopsin and various ^{13}C labels; (right traces) the corresponding subtractions are shown whereby the spectrum of the unmodified pigment was subtracted from the spectra of the labeled one.

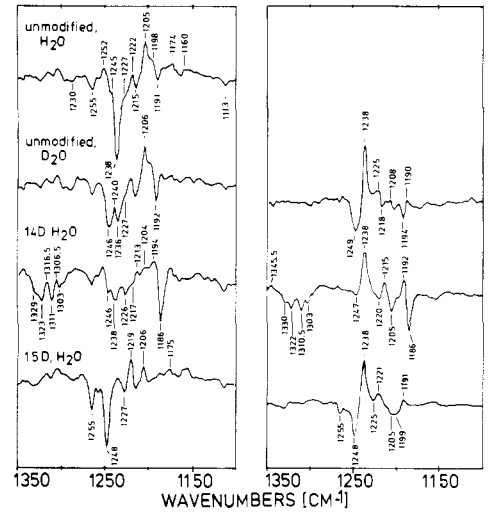

FIGURE 8: Rhodopsin–lumirhodopsin difference spectra in the region of the C–C stretching vibrations. (Left traces) Unmodified rhodopsin, H_2O; unmodified rhodopsin, ^2H_2O; C_{15}-^2H-labeled rhodopsin, H_2O; C_{14}-^2H-labeled rhodopsin, H_2O; (right traces) the corresponding subtractions are shown whereby the spectrum of the unmodified pigment measured in H_2O was subtracted from the spectra of the labeled one.

cm^{-1} (Figure 6). The rhodopsin line at 1390.5 cm^{-1} gains intensity in the spectrum of the C_{15}–^2H derivative and is shifted to 1391.5 cm^{-1} as can be seen in the subtraction. In contrast to the unmodified pigment, this 1391.5 cm^{-1} rhodopsin line is insensitive to $H/^2H$ exchange of the medium. In the C_{15}–^2H pigment, new rhodopsin and lumi bands appear at 1366 and 1360 cm^{-1}, respectively. Additionally, a new broad rhodopsin line appears at 1332 cm^{-1} which is shifted in ^2H_2O to 1329.5 cm^{-1}.

Deuteriation at the position of C_{14} (Figure 5C,D) causes new rhodopsin lines at 1378, 1329, 1323, 1311, and 1303 cm^{-1}, and the 1390.5 cm^{-1} line of unmodified rhodopsin disappears. A new lumirhodopsin line appears at 1447 cm^{-1} in H_2O and disappears in ^2H_2O. Another new lumirhodopsin line appears at 1344 cm^{-1} in H_2O and 1354 cm^{-1} in ^2H_2O. The C_{14}–^2H bending mode of lumirhodopsin can be assigned to the new line at 969.5 cm^{-1} overlapping the two rhodopsin lines at 972 and 968 cm^{-1}. The corresponding rhodopsin line absorbs at 937.5 cm^{-1} (Figure 6).

To assign the C–C stretching vibrations between 1250 and 1100 cm^{-1}, the subtraction of the spectra is useful. Figures 7 and 8 show the difference spectra of unlabeled and labeled rhodopsin and the corresponding subtraction. Bands of modified rhodopsin are at 1290, 1265, and 1238 cm^{-1} with a shoulder at 1227, 1215, and 1191.5 cm^{-1}. Lines of unmodified lumirhodopsin are observed at 1252, 1222.5, and 1205.5 cm^{-1} with a shoulder at 1198, 1174, and 1160 cm^{-1}.

Palings et al. (1987) assigned the C_8–C_9 stretch of rhodopsin to a line at 1216 cm^{-1}. This is consistent with our results. In the difference spectrum of rhodopsin regenerated with [C_{19}-$^2H_3$]retinal (Figure 7), the 1215 cm^{-1} peak disappears, and, instead, a lumirhodopsin line is observed at the same wavenumber. Also, the main lumirhodopsin line at 1205.5 cm^{-1} disappears by this labeling. In the subtraction, two large lines are observed: a positive line at 1216 cm^{-1} due to the C_8–C_9 stretch vibration of the unmodified rhodopsin which shifts to 1206 cm^{-1} in the C_{19}-$^2H_3$ derivative. As we do not observe the corresponding lumirhodopsin C_8–C_9 stretches, we conclude that they also absorb at 1216 and 1206 cm^{-1} but with less intensity so that only the more intense rhodopsin line can be observed in our difference spectra. The assignment of the C_8–C_9 stretching mode to 1214 cm^{-1} in the resonance Raman spectra of bathorhodopsin by Palings et al. (1987) supports our conclusion.

The C_{14}–C_{15} stretching vibration of rhodopsin can be assigned to the 1191.5 cm^{-1} line due to its shift on ^{13}C labeling (Figure 7). In the 14-^{13}C-labeled pigment, the line shifts to 1183 cm^{-1}, in 15-^{13}C to 1185 cm^{-1}, and in the 14,15-$^{13}C_2$ derivative to 1171 cm^{-1}. This assignment of the C_{14}–C_{15} stretching vibration is also in agreement with the resonance Raman results of Palings et al. (1987). The 1191.5 cm^{-1} line is sensitive to ^{13}C substitution at the C_{10}- and C_{11}-positions as seen by its shift to 1186 cm^{-1} (Figure 7). This may be caused by a small coupling of the C_{10}–C_{11} and the C_{14}–C_{15} stretches. The C_{14}–C_{15} stretch in lumirhodopsin can be assigned to the line at 1205.5 cm^{-1}. In the 14-^{13}C pigment, this line shifts to 1190 cm^{-1}, in the 15-^{13}C pigment to 1192 cm^{-1}, and in the 14,15-$^{13}C_2$ pigment to 1176.5 cm^{-1}. In all three spectra, a line remains at 1203 cm^{-1}. On 10,11-$^{13}C_2$ labeling, the 1206 cm^{-1} line shifts a few wavenumbers to 1201.5 cm^{-1}.

The 1205.5 cm^{-1} lumirhodopsin line and the 1191.5 cm^{-1} rhodopsin line disappear in the spectrum of the C_{15}-^2H derivative, and new lines appear at 1219 cm^{-1} for lumirhodopsin and at 1199 cm^{-1} in rhodopsin (Figure 8). In the spectra of rhodopsin deuteriated at C_{14}, both 14,15 stretching vibrations disappear. The rhodopsin line seems to be downshifted to 1186 cm^{-1} and gains intensity. A new lumirhodopsin line appears at 1213 cm^{-1} (Figure 8).

The rhodopsin C_{10}–C_{11} stretching vibration itself is expected at 1100 cm^{-1} (Palings et al., 1987). In our difference spectra, we cannot assign any line in this spectra region to the C_{10}–C_{11} stretch. The C_{10}–C_{11} lumirhodopsin line is expected at 1160 cm^{-1} (Smith et al., 1985; Palings et al., 1987). In the subtraction, a negative band at 1159 cm^{-1} and a small positive band at 1143 cm^{-1} can be seen (Figure 7). Therefore, we assign the 1159 cm^{-1} line to the C_{10}–C_{11} stretching vibration of lumirhodopsin and the 1143 cm^{-1} band to the shifted one.

The assignment of the 1238 cm^{-1} rhodopsin line is of some controversy. In the FTIR difference spectra, this line is the most intense one. The line is sensitive to ^{13}C isotopic labeling at the C_{14}- and C_{15}-positions (Figure 7). In the 15-^{13}C derivative, the 1238 cm^{-1} line is downshifted to 1233 cm^{-1} and loses intensity. The line at 1265 cm^{-1} gains intensity. The same charcteristics are observed for the 14-^{13}C pigment. The 1238 cm^{-1} line is downshifted to 1234.5 cm^{-1} and loses intensity while the intensity of the 1265 cm^{-1} line is increased. On 14,15-^{13}C substitution, the line is downshifted to 1230.5 cm^{-1}, and the 1264 cm^{-1} line gains intensity. For the C_{15}-^{2}H pigment (Figure 8), the 1238 cm^{-1} line is upshifted to 1248 cm^{-1}. Upon C_{14}-^{2}H substitution (Figure 8), most of the 1238 cm^{-1} line disappears, revealing three lines at 1247 and 1277 cm^{-1} and a small line remaining at 1238 cm^{-1}. The line is also sensitive to medium deuteriation (Figure 8), which shifts part of the line up to 1247 cm^{-1} with a remaining line at 1235 cm^{-1}. After ^{13}C or deuterium substitution at C_{14} and C_{15}, no additional effect is observed after the $H/^{2}H$ exchange reaction.

The hydrogen out of plane vibrations absorb in the region below 1000 cm^{-1}. In contrast to the bathorhodopsin lines, the HOOPs of lumirhodopsin appear only with low intensity (Figure 1).

Discussion

In the rhodopsin–lumirhodopsin difference spectra, we observe several carbonyl stretching vibrations. A difference line appears at 1772.5 cm^{-1}/1767 cm^{-1}. The same line was previously observed in the rhodopsin–bathorhodopsin spectrum and is thought to be caused by a protonated internal aspartic acid or glutamic acid side chain which is not hydrogen bonded (Siebert et al., 1983; Bagley et al., 1985). The lumirhodopsin line at 1741 cm^{-1} and the rhodopsin line at 1734 cm^{-1} are also sensitive to $H/^{2}H$ exchange. Because of the lower absorption frequency, we assign these lines to a weakly hydrogen-bonded carboxylic side chain of which the environment is changed during the phototransition. However, a protonation of one carboxylic group and a deprotonation of another one cannot be excluded.

The rhodopsin line at 1725 cm^{-1} was previously observed by de Grip et al. (1985) and discussed as a carboxylic group which does not undergo $H/^{2}H$ exchange because it is buried in the hydrophobic part of the protein. Because of the rapid $H/^{2}H$ exchange reaction of the buried carboxylic group absorbing at 1772.5 cm^{-1}/1767 cm^{-1}, we would expect the same for this group. In the structure proposed by Ovchinnikov (1982, 1986), only three internal acid residues are suggested at positions 83, 113, and 122. In the model proposed by Hargrave et al. (1983), the Glu-113 is assigned to the external part of the protein and Glu-134 to the internal part. However, if we assume a carboxylic anion as the counterion of the protonated Schiff base nitrogen and the carbonyl vibration at 1725 cm^{-1} as caused by an acid side chain, four carboxyl groups would be located in the internal part of the protein. This also argues against an assignment of the 1725 cm^{-1} band to a carboxylic C=O stretching vibration.

Because of their high frequencies, the 1725 cm^{-1} rhodopsin line and the 1731.5 cm^{-1} lumirhodopsin line cannot be assigned to the amide carbonyl stretching vibration of an asparagine or a glutamine residue. Primary hydrogen-bonded amides absorb around 1650 cm^{-1}, and the bands shift up to 1690–1700 cm^{-1} in the monomeric form in hydrophobic solvents (Richards & Thompson, 1947). Such a hydrophobic environment would appear as a reasonable assumption, since the carboxylic band at 1767 cm^{-1} is well matched by the absorption of monomeric acetic acid in CCl_4 (Grove & Willis, 1951).

Therefore, we propose a different explanation for the 1725 cm^{-1}/1731.5 cm^{-1} lines. The low frequency of the C=O stretching vibration of common peptide bonds is mainly due to the resonance between the π electrons of the C=O bond and the lone pair of the amide nitrogen, whereby the C=O bond strength is lowered and the corresponding C–N bond is strengthened. This results in the coplanarity of the amide group and the large barrier for rotation around the C–N bond and is explained by assuming an sp2 hybridization for the nitrogen atom. If this resonance is not possible, a large upshift of the C=O stretching frequency of the amide is observed. In *N*-acetylpyrrole, the lone pair of the nitrogen takes part in the aromatic system of the pyrrole ring, thereby shifting the C=O stretching vibration to 1737 cm^{-1} (Otting, 1956). In *N*-acetylaziridine (Spell, 1967) and the four-membered lactam ring of the penicillins (Bellamy, 1966), the geometry of these small rings is not compatible with the requirements of an sp2 hybridization of the amide nitrogen which also results in an upshift of the C=O stretching vibration to 1707 and 1740–1760 cm^{-1}, respectively. If the C–N bond were twisted, a similar large increase of the C=O frequency would be expected.

For a normal peptide bond, the rotational barrier amounts to 30 kcal/mol, impeding larger distortions. However, for an amino acid bound to the N-terminal end of a proline, the barrier is only 13 kcal/mol, allowing even cis–trans isomerization (Schulz & Schirmer 1979). Therefore, it is conceivable that steric hindrance between the retinal moiety and the protein will result in a twist of the C–N–H group of the proline out of the plane of the adjacent carbonyl bond and will upshift the amide I vibration to 1725 cm^{-1}. In lumirhodopsin, this bond is further distorted, thereby upshifting to 1731.5 cm^{-1}.

Because both species, rhodopsin and lumirhodopsin, exhibit similar absorption maxima in the visible, the C=N stretches of the Schiff base are expected to absorb at the same frequency. Our results show that the C=N stretch is 23 cm^{-1} lower in lumirhodopsin as compared to rhodopsin. This difference must be due to a much stronger coupling (Aton et al., 1980; Smith et al., 1985; Kakitani et al., 1983) of the C=N stretch with the N–H and C_{15}–H bending vibration in rhodopsin as compared to lumirhodopsin. This is supported by the observation that the C=N mode in $^{2}H_2O$ shifts down by 36 cm^{-1} in rhodopsin but only by 4 cm^{-1} in lumirhodopsin. Substitution of C_{15}–^{2}H also results in a larger shift in rhodopsin (20 cm^{-1}) as compared to lumirhodopsin (10 cm^{-1}). Because the C=N stretching frequency was observed at nearly the same position in rhodopsin and bathorhodopsin (Siebert et al., 1983; Palings et al., 1987), it was suggested that the environment of the Schiff base is only slightly changed during the rhodopsin–bathorhodopsin transition. However, on cis–trans isomerization, the Schiff base moiety should change its position. Palings et al. (1987) explained this discrepancy by assuming that the twists along the single and double bonds of the polyene chain, causing the intense HOOP vibrations in bathorhodopsin, co-add so that the Schiff base remains in the same environment. This is consistent with our results. In lumirhodopsin, no intense HOOP vibrations are observed, and the C=N stretching frequency and its coupling behavior are altered, suggesting a different environment in lumirhodopsin as compared to rhodopsin.

Recently, an interesting point was raised by Lopez-Garriga et al. (1986a–c). Performing careful quantum chemical calculations and normal mode analysis, they showed that protonation of Schiff bases rather increases the C=N force constant, being the primary cause for the increase of the C=N

stretching frequency. This is in contrast to the C=/N–H coupling model mentioned above. By suitable chosen force constants also in their model, large isotopic shifts on deuteriation can be observed. Because the C=N stretching frequency of this model is also very sensitive to hydrogen bonding of the Schiff base's proton, our basic conclusions on changes of the environment of the Schiff base with the formation of lumirhodopsin are not altered by these considerations.

The other lines observed in the Schiff base region of lumirhodopsin and rhodopsin must be due to the protein. The lines may be caused by alteration of amide I vibrations of the peptide backbone. The intense 1655 cm^{-1} line in rhodopsin could be explained by the ring vibration of the indole ring of a tryptophan residue. On the basis of UV–vis spectra, Rafferty (1979) suggested that a tryptophan side chain is involved in the photoreaction.

In the rhodopsin–bathorhodopsin and rhodopsin–isorhodopsin difference spectra (Bagley et al., 1985; Siebert et al., 1983; Rothschild et al., 1983), the rhodopsin ethylene modes were assigned to lines at 1562, 1555, and 1547 cm^{-1}. Since the strong rhodopsin lines cannot be seen in the spectrum, they must be mostly hidden under the more intense lumirhodopsin lines. In the spectrum of the unmodified rhodopsin, only a rhodopsin line at 1550 cm^{-1} is observed, which is probably due to the 1547 cm^{-1} band. The 1526 cm^{-1} line is probably not an ethylenic mode. Due to the red-shift of the absorption maximum (540 nm versus 498 and 492 nm), the ethylenic modes of bathorhodopsin are shifted down relative to those of rhodopsin and lumirhodopsin, thereby unveiling the vibration of rhodopsin.

In contrast to RR spectra where only one intense line appears for unmodified rhodopsin and bathorhodopsin (Palings et al., 1987), we observe more lines of comparable intensity for both rhodopsin and lumirhodopsin. In the spectra of ^{13}C-labeled rhodopsin (Palings et al., 1987), more lines are observed which are upshifted to approximately 1560 cm^{-1}, indicating that more ethylenic modes are present. It is interesting to note that FTIR spectra of bacteriorhodopsin also exhibit only one intense ethylenic band. Therefore, the charge distribution along the chain must be different in lumirhodopsin and bacteriorhodopsin.

The ethylene modes are not sensitive for ^{13}C substitution at C_{15}. Since deuteriation at the C_{15}-position alters the spectrum, we conclude that the C_{15}–H bending mode is coupled to the ethylene stretching vibrations. The lumirhodopsin line at 1542 cm^{-1} is sensitive to isotopic substitution at C_{14}, C_{10}, C_{11}, and C_9. Thus, the band must be caused by a combination of these stretching modes. However, generally, due to the overlap of the ethylene modes of rhodopsin and lumirhodopsin, it is difficult to derive the shifts produced by the different labels.

The rhodopsin line at 1456 cm^{-1} disappears on C_{15}–^2H substitution and is also sensitive to $H/^2H$ exchange as indicated by its upshift of 4 wavenumbers to 1460 cm^{-1} in ^2H_2O. We assign this line to the C_{15}–H bending mode of rhodopsin, 100 wavenumbers higher than observed for model compounds (Smith et al., 1986). This large upshift must be caused by the protein. The 1456 cm^{-1} line is only slightly shifted on deuteriation of the Schiff base nitrogen, so we have to conclude that the N–H and the C_{15}–H are nearly decoupled. This finding is strikingly different from model substances and theoretical calculations (Smith et al., 1985). Because the 1456 cm^{-1} line is present in the spectrum of C_{14}-^2H-modified rhodopsin, the C_{15}–H bending mode is also not coupled to the C_{14}–H rock. The 1456 cm^{-1} line is slightly shifted in the 14,15-^{13}C-labeled rhodopsin which further supports our assignment. The C_{15}–^2H bend is observed at 934.5 cm^{-1} in lumirhodopsin as expected from theoretical calculations (Smith et al., 1985), but the rhodopsin line is 80 cm^{-1} upshifted and observed at 1012 cm^{-1}. The same holds true for bathorhodopsin where the band is located at 1010 cm^{-1} (our own unpublished results). This again indicates the similarity of the environment of the Schiff bases in rhodopsin and bathorhodopsin and the large changes occurring in lumirhodopsin.

In [C_{15}-^2H]rhodopsin, we observe an upshift of a line from 1356 to 1366.5 cm^{-1} which is probably due to another C–H bending mode coupled to the C_{15}–H vibration. Deuteriation at the C_{15}-position removes the coupling, and a small upshift is observed. The same effect is visible in the RR spectra published by Eyring et al. (1982). The N–H bending vibration of rhodopsin is calculated to be located around 1350 cm^{-1} (Bagley et al., 1985). We observe in the frequency range of 1500–1250 cm^{-1} many small changes on $H/^2H$ exchange but cannot assign any of these lines unequivocally to the N–H bending mode. On regeneration of rhodopsin with either [C_{15}-^2H] or [C_{14}-^2H]retinal, the effects of $H/^2H$ exchange are less pronounced, indicating that the N–H bend mode is distributed over several modes.

In agreement with FTIR investigations of the rhodopsin–bathorhodopsin transition (Bagley et al., 1985; our own unpublished results), the negative band at 1390.5 cm^{-1} in the rhodopsin–lumirhodopsin difference spectrum, of unmodified rhodopsin, disappears in ^2H_2O, but in the C_{15}-^2H derivative, it is not sensitive to deuteriation of the medium. In the spectra of C_{14}-^2H-modified rhodopsin, the 1390.5 cm^{-1} line disappears, and a new line appears at 1378 cm^{-1}. This line must be due to a combination of the N–H, C_{15}–H, and C_{14}–H bending modes most likely coupled to another bending mode. Another possibility is that the 1390.5 cm^{-1} line is caused mainly by the C_{14}–H rock and a new mode appears at 1378 cm^{-1}. In the resonance Raman spectra (Palings et al., 1987), the 1238 cm^{-1} line is insensitive to $H/^2H$ exchange. The line was assigned to the C_{12}–C_{13} stretching vibration mainly because of its disappearance on deuteriation at C_{14}. Because we too observe the disappearance of the 1238 cm^{-1} line in the C_{14}–^2H pigment and because of the other observed shifts described above, we assign this line to a mode consisting of the C_{12}–C_{13} and C_{14}–C_{15} stretching vibrations and the N–H, C_{15}–H, and C_{14}–H bending vibrations. Deuteriation at N, C_{14}, or C_{15} removes the coupling with the respective bending modes and causes an upshift. The coupling of the C_{15}–H bending mode is also present in the RR spectra published by Eyring et al. (1982). They observed an upshift of the 1238 cm^{-1} line to 1250 cm^{-1} as a shoulder at the 1265 cm^{-1} line. In the RR spectrum, the line is not sensitive to $H/^2H$ exchange at the Schiff base nitrogen. This slight difference between the modes observed in resonance Raman spectrum and the infrared spectrum is due to the different spectroscopic methods.

The C_{10}–C_{11} single-bond stretching vibration in lumirhodopsin is assigned to 1159 cm^{-1} due to its shift on 10,11-^{13}C labeling. Figure 9 shows that in bathorhodopsin the C_{10}–C_{11} stretching vibration is located at 1166 cm^{-1}. This is in agreement with the tentative assignment of Bagley et al. (1985) and with the direct assignment of Palings et al. (1987). These two groups concluded from the high position that the geometry in bathorhodopsin must be 10-s-trans. The increase of the C_8–C_9 stretching frequency as compared to that of the all-trans PSB model substance shows that the electronic structure is more delocalized, extending up to the C_8–C_9 single bond (Palings et al., 1987). According to the arguments of Tavan

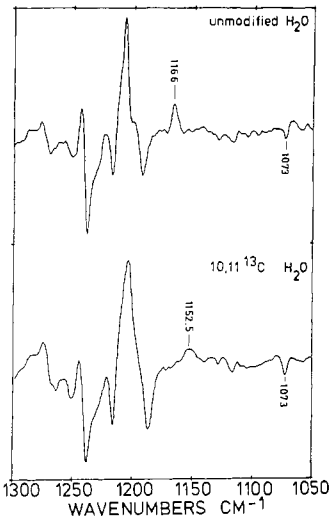

FIGURE 9: Rhodopsin–bathorhodopsin difference spectra in the region of the C–C stretching vibrations. (Upper trace) Unmodified rhodopsin, H_2O; (lower trace) 10,11-^{13}C-modified rhodopsin, H_2O.

and Schulten (1986), the unequivocal deduction of the geometry could be questioned. Therefore, we use the frequencies of lumirhodopsin, representing an all-trans PSB within the protein, as a reference system, which partly takes into account the influence of the protein. Despite the large red-shift of the absorption maximum as compared to the all-trans PSB model substance (490 versus 440 nm), the C_{10}–C_{11} stretching vibration is at the same position (Smith et al., 1985). Preliminary results suggest a further downshift in the meta I photoproduct to 1156 cm^{-1}. Since the electronic distortion in bathorhodopsin is not as large as in the K_{610} intermediate of bacteriorhodopsin, a 10,11-s-cis bond would result in a downshift of at least 20 cm^{-1} as compared to lumirhodopsin (Tavan & Schulten, 1986). Therefore, also if the arguments of Tavan and Schulten (1986) are taken into consideration, a 10,11-s-cis bond in bathorhodopsin can be excluded. Our results show that electron delocalization in bathorhodopsin does not invalidate the original conclusions of Bagley et al. (1985) and Palings et al. (1987). Bagley et al. (1985) assigned a band at 1073 cm^{-1} to the C_{10}–C_{11} mode of rhodopsin. Since 10,11-^{13}C labelling does not produce a shift of this band (Figure 9), this assignment can be excluded.

In the resonance Raman spectra, the intensity of the HOOPs is well correlated to the out of plane twist of the polyene chain (Eyring et al., 1982). In the infrared, the same behavior was observed, but no theoretical explanation is available as yet. Because the HOOPs show only low intensity, we conclude that the constraints that are present in the bathorhodopsin species must be relaxed in lumirhodopsin.

CONCLUSIONS

In contrast to the small molecular changes of the protein observed in the rhodopsin–bathorhodopsin difference spectra, larger changes occur in the bathorhodopsin–lumirhodopsin transition. They are most easily detected in the region between 1800 and 1600 cm^{-1}. In the rhodopsin–lumirhodopsin difference spectra, two carboxylic groups are detected due to their shift in ^2H_2O medium. A third carbonyl stretching vibration is tentatively assigned to the amide I of an amino acid bound to a twisted proline residue.

The Schiff base stretching vibrations of rhodopsin and lumirhodopsin can be assigned. The rhodopsin line is, for the first time, directly observed in the infrared. The assignment at 1659 cm^{-1} is in agreement with the resonance Raman results. The assignment of the C=N stretch for rhodopsin and lumirhodopsin together with its isotopic shifts and with the observed in-plane bending vibrations allows for some interesting molecular interpretations. The high position (1659 cm^{-1}) is the same for rhodopsin and bathorhodopsin as are, approximately, the large isotopic shifts produced by deuteriation at N and C_{15}. They indicate that the C=N is strongly coupled to the bending modes, more than observed for other retinylidene Schiff bases. Also, the C_{15}–H and C_{15}–^{2}H bending vibrations show unusually high frequencies, demonstrating that the coupling behavior is identical in rhodopsin and bathorhodopsin (Siebert et al., 1983; Palings et al., 1987) and that the environment must essentially remain the same. A completely different behavior is observed for lumirhodopsin. Here, the C=N stretch is only weakly coupled to the bending vibrations, and the C_{15}–^{2}H bending modes are observed in the normal frequency range. Thus, the environment of the Schiff base region must be altered, which, finally, will result in the deprotonation of the Schiff base in meta II.

In the spectral region of the C–C stretching vibrations, the infrared spectrum agrees reasonably well with the resonance Raman data for rhodopsin. Only at 1238 cm^{-1}, we see a mode consisting of the C_{12}–C_{13} and C_{14}–C_{15} stretching vibrations and of the N–H and C_{15}–H bending vibrations. This mode may also be caused by the perturbation of the Schiff base in rhodopsin. The C_{10}–C_{11} single-bond stretch in lumirhodopsin is assigned to 1159 cm^{-1} due to its shift on 10,11-^{13}C labeling. Together with the assignment of the corresponding band in bathorhodopsin, we can exclude a C_{10}–C_{11} s-cis bond in bathorhodopsin.

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Isolation and Purification of an Fc_ϵ Receptor Activated Ion Channel from the Rat Mast Cell Line RBL-2H3†

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ABSTRACT: Derivatives of the antiallergic drug cromolyn [disodium 5,5′-[(2-hydroxy-1,3-propanediyl)-bis(oxy)]bis[4-oxo-(4*H*-1-benzopyran)-2-carboxylate]], which can be conjugated covalently at the propane 2-position to macromolecules and to insoluble matrices, were synthesized. Conjugates of these derivatives with macromolecules were examined for their binding to cells of the rat basophilic leukemia line RBL-2H3, which is widely employed as a model for immunologically induced mast cell degranulation. Only those drug–protein conjugates in which the cromolyn analogue with an amino group at the propane 2-carbon instead of the hydroxyl was linked to the carrier by glutaraldehyde were found to exhibit specific and saturable binding to these cells. Analysis of the binding data for these conjugates yielded an apparent binding constant of $3.8 \pm 0.2 \times 10^8$ M^{-1} and an apparent number of binding sites for the probe of 4000–8000 per cell. The conjugates found to bind specifically to the cells were also immobilized on agarose matrices and employed in an affinity-based isolation of the membrane component responsible for the observed binding. A single labeled polypeptide was eluted from these columns, onto which either whole cell lysates or solubilized purified plasma membranes of surface-radioiodinated RBL-2H3 cells had been adsorbed. This membrane protein appears on autoradiograms of nonreducing SDS–PAGE as a single broad band of ~110 000 daltons (Da) apparent molecular mass. On autoradiograms of reducing gels, the only band detected has an apparent mass of ~50 000 Da and appears narrower. Elution of the columns with the drug and disulfide-reducing agents or with the latter alone resulted in significantly higher yields of the 50-kDa polypeptide. Both the intact and reduced proteins bind strongly to immobilized concanavalin A and less so to immobilized wheat germ agglutinin, suggesting that the isolated intact protein is probably a dimer of two glycosylated subunits of similar molecular mass. Treatment of the reduced protein with endoglycosidase F leads to a decrease in its apparent molecular mass by ~12 kDa, suggesting that the extent of glycosylation of this polypeptide is ~25%. As shown in the following paper, the intact protein constitutes a Ca^{2+} channel that is activated upon IgE–Fc_ϵ receptor aggregation.

The nature and sequence of biochemical processes, which couple the IgE-mediated stimulus with mediator secretion from mast cells, are topics of considerable research interest and activity (Ishizaka & Ishizaka, 1984; Gomperts & Fewtrell, 1985). One of the early events in this cascade that has been intensively studied and discussed is the transient increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mast cells of different origins (White et al., 1984; Beaven et al., 1984; Pervin et al., 1985; Sagi-Eisenberg et al., 1985). The requirement for millimolar concentrations of Ca^{2+} in the extracellular medium for the immunologically triggered secretion had been widely documented already in early studies (Mongar & Schild, 1958; Lichtenstein & Osler, 1964; Greaves & Mongar, 1968). Furthermore, different experiments provided indications for a net influx of these ions down their concentration gradient into the cells, probably via ion channels (Foreman & Mongar, 1972; Foreman et al., 1977a). In recent

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