

Evidence for light-induced 13-*cis*, 14-*s-cis* isomerization in bacteriorhodopsin obtained by FTIR difference spectroscopy using isotopically labelled retinals

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Communicated by D.Oesterhelt

We have obtained by Fourier transformed infra-red (FTIR)-spectroscopy BR-K, BR-L and BR-M difference spectra of bacteriorhodopsin regenerated with isotopically labelled retinals. Thereby, we are able to assign reliably the C₁₄–C₁₅ and C=N stretching vibrations of the various intermediates. The lower C₁₄–C₁₅ stretching vibration frequency in L as compared with 13-*cis* protonated Schiff base model compounds indicates a 13-*cis*, 14-*s-cis* configuration of the retinal in this species. The unusually low C=N stretching vibration in K at 1615 cm⁻¹ indicates less stabilization of the positive charge at the Schiff base by the protein environment. Based on these results, a mechanism is suggested by which the stored light energy is transformed into proton transfers.

Key words: FTIR/bacteriorhodopsin/vibrational analysis/proton pump mechanism/retinal isotopic derivatives

Introduction

The retinal-protein bacteriorhodopsin (BR) transduces light-energy into electrochemical energy by a proton transfer across the membrane (Oesterhelt and Stoekenius, 1973). To understand the mechanism by which the light-energy is transformed into proton transfers, we investigated the molecular events in the chromophore and the chromophore–protein interaction during the photocycle of BR₅₆₈ (BR). The initial photochemical step in this photocycle involves an all-*trans* to 13-*cis* isomerization around the C₁₃=C₁₄ double bond of the retinal, leading to K₆₁₀ (K), which decays thermally back to BR through the intermediates L₅₅₀ (L), M₄₁₂ (M) and O₆₄₀ (O) (Ottolenghi, 1980; Stoekenius and Bogomolini, 1982).

Recently, we have shown that four internal aspartic acids undergo protonation changes during the photocycle of BR (Engelhard *et al.*, 1985). Thereby the different absorption maxima of the intermediates could be explained, assuming the position of the aspartic acids to be near the protonated Schiff base (PSB) — the binding site between chromophore and protein. In the L to M transition the pK of the Schiff base nitrogen seems reduced and thus the Schiff base proton can be transferred most likely to an aspartate named asp(1). In the proposed model the pK-reduction of the protonated Schiff base is caused by a hindered 14-*s-cis* configuration, as originally suggested by Schulten and Tavan (1978). Nevertheless, the experimental proof for a 14-*s-cis* configuration during the photocycle is still missing.

To determine the isomerization states of the C₁₄–C₁₅ bond and the electron distributions in the Schiff base region we investigated, by FTIR spectroscopy, the photocycle of BR regenerated with labelled retinals. By using 10-11-di-¹³C-, 14-¹³C-, 15-¹³C-, 14-15-

di-¹³C-, 14-²H- and 15-²H-labelled retinals, we assigned in the i.r. spectra most of the bands arising from vibrations of the terminal part of the chromophore. A basis for the assignments was given by vibrational analyses of retinals and retinal PSB model compounds (Braiman and Mathies, 1980; Curry *et al.*, 1982, 1984; Saito and Tasumi, 1983; Smith *et al.*, 1985a). The lower frequency of the C₁₄–C₁₅ stretching vibration at 1155 cm⁻¹ in L as compared with the 13-*cis* retinal PSB model compound indicates a 14-*s-cis* configuration. The unusually low frequency of the C=N stretching vibration in K at 1613 cm⁻¹ must be attribu-

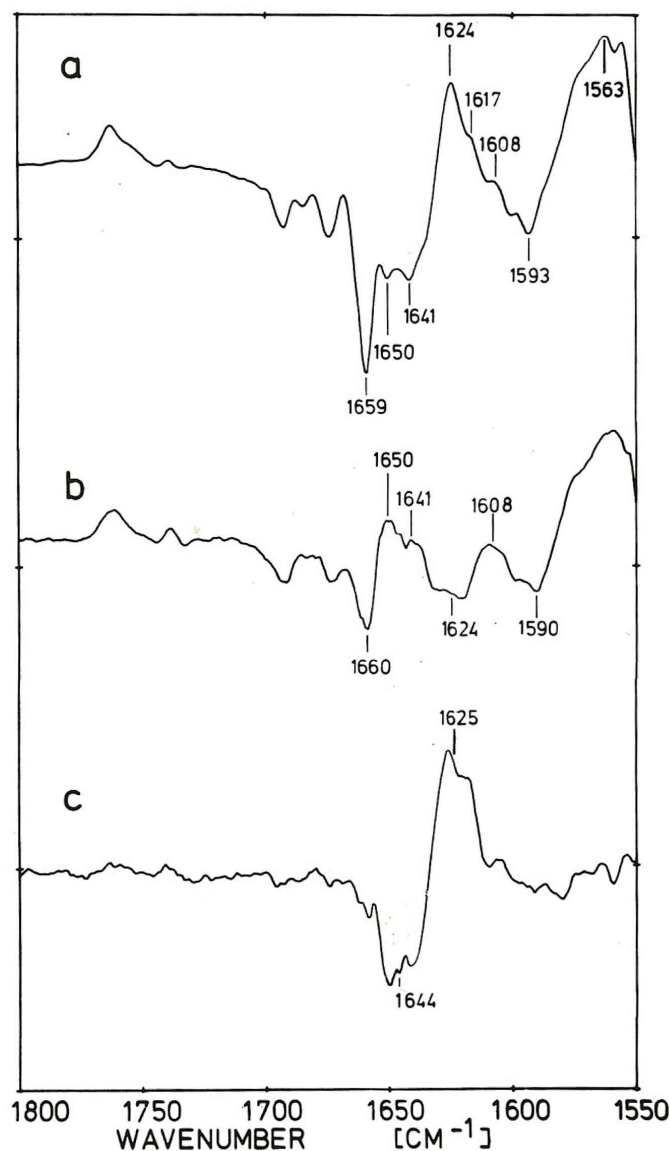


Fig. 1. BR-M difference spectra in the Schiff base region for unlabelled (a) and 15-¹³C-labelled (b) compounds in H₂O. The sum of the subtractions is shown in (c).

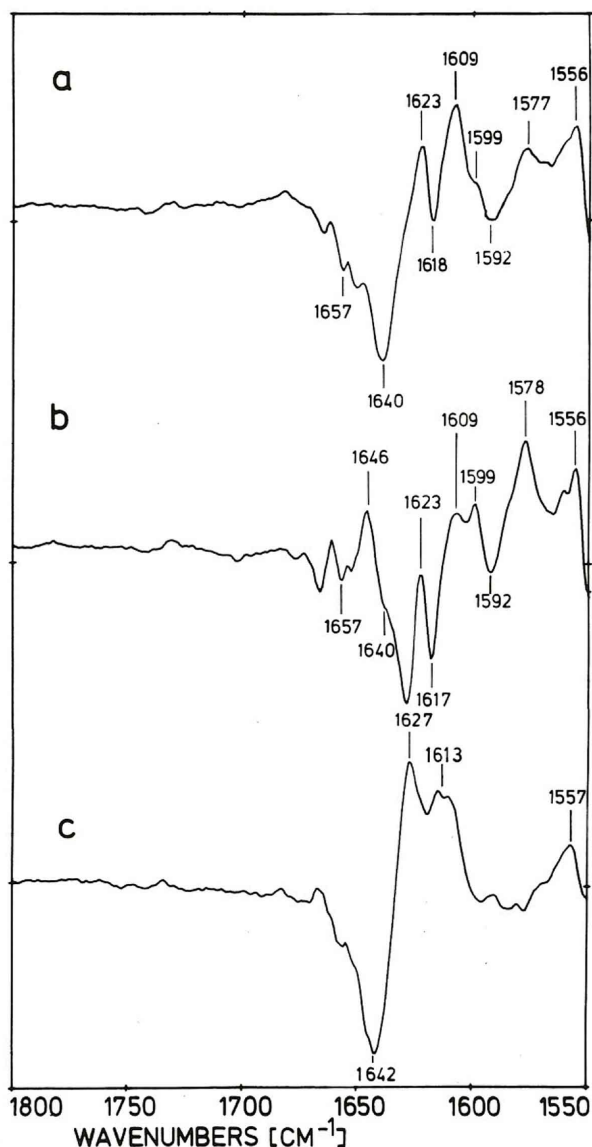


Fig. 2. BR-K difference spectra in the Schiff base region for unlabelled (a) and $15\text{-}^{13}\text{C}$ -labelled (b) compounds in H_2O . The sum of the subtractions is shown in (c).

ted, as shown by vibrational analysis, mainly to a decrease of the $\text{C}=\text{N}$ bond order.

Results

Figure 1 (a and b) shows the BR-M difference spectra in the region in which the $\text{C}=\text{N}$ stretching vibration of the Schiff base is expected to be observed. Because many protein bands are present, it is difficult to identify the shift of the BR $\text{C}=\text{N}$ stretching vibration band in the spectrum of the $15\text{-}^{13}\text{C}$ -labelled compound. To obtain a reliable assignment of the shifted band, we subtracted both spectra as described in Materials and methods (Figure 1c). By this subtraction, those bands which are not influenced by the labelling are cancelled. In order to check the reliability of the subtraction, we subtracted three labelled and unlabelled BR-M-spectra, respectively. Only those features of the subtraction are taken as real which are reproduced in the individual subtractions. The small peaks superimposed on the broad bands do not meet this criterion. Thus we assigned the centre

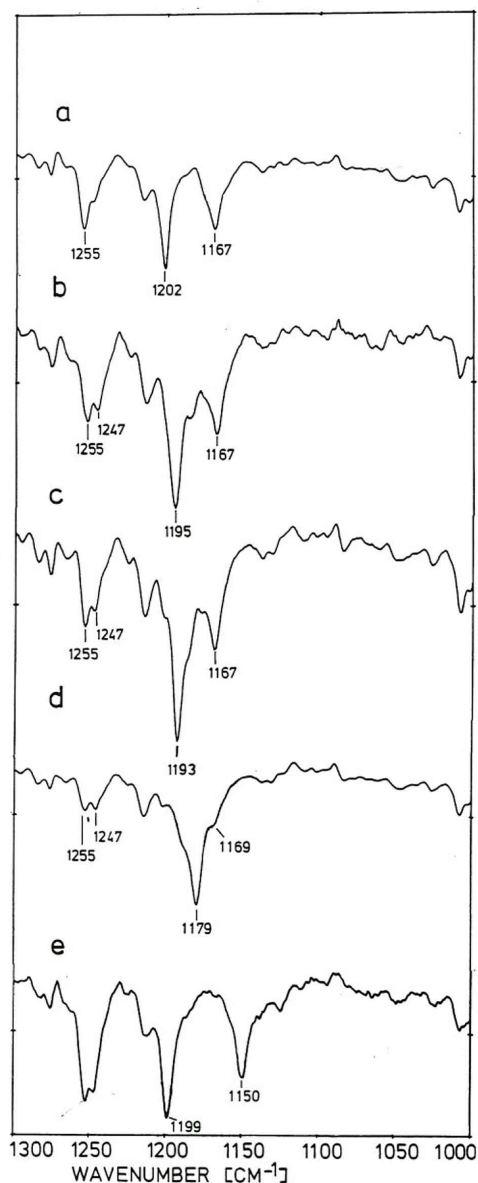


Fig. 3. BR-M difference spectra in the fingerprint region for unlabelled (a), $15\text{-}^{13}\text{C}$ -labelled (b), $14\text{-}^{13}\text{C}$ -labelled (c), $14\text{-}15\text{-di}^{13}\text{C}$ -labelled (d) and $10\text{-}11\text{-di}^{13}\text{C}$ -labelled (e) compounds.

of the negative band at about 1644 cm^{-1} , in general agreement with the $\text{C}=\text{N}$ stretching vibration of BR, and the centre of the positive band at 1625 cm^{-1} to the shifted band. This demonstrates that the subtraction provides reliable assignments which would have been difficult to make directly in the difference spectra. The course of the base line between 1700 cm^{-1} and 1800 cm^{-1} is a measure of the variation in the different subtractions.

Figure 2 (a and b) shows the BR-K difference spectra in the Schiff base region. In the subtraction (Figure 2c) it is indicated, in addition to the shift of the negative BR $\text{C}=\text{N}$ band from 1642 cm^{-1} to 1627 cm^{-1} , that a positive band at about 1613 cm^{-1} is shifted. Based on the criteria developed above, the small peaks are not taken as real. Therefore, we assign the band at 1613 cm^{-1} to the $\text{C}=\text{N}$ stretching vibration of K. From the subtraction it is not clear to where this band shifts. There is only a broad, weak, negative band at about 1580 cm^{-1} . But, in the

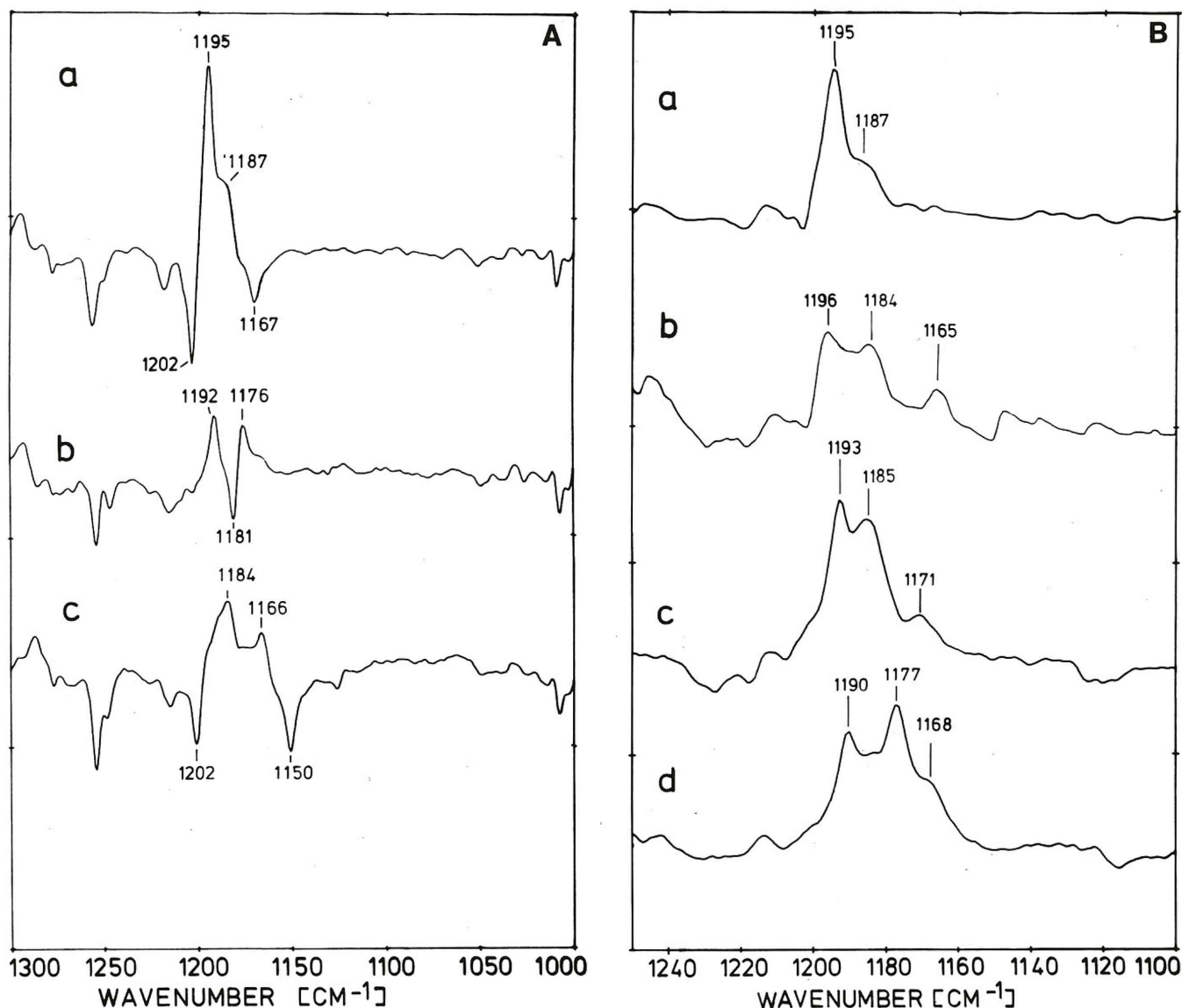


Fig. 4. (A) BR-K difference spectra in the fingerprint region for unlabelled (a), 14-15-di- ^{13}C -labelled (b) and 10-11-di- ^{13}C -labelled (c) compounds. (B) The K bands, obtained by subtracting the BR bands as described in Materials and methods, are shown for unlabelled (a), 10-11-di- ^{13}C -labelled (b), 14- ^{13}C -labelled (c) and 14-15-di- ^{13}C -labelled (d) compounds.

spectrum of the 15- ^{13}C -labelled compound (Figure 2b), the band at 1578 cm^{-1} gains intensity. Since the gain of integral intensity is not as large as the loss at about 1613 cm^{-1} , the shifted band is less clearly reflected in the subtraction of the spectra. The intensity of the shifted band could be obscured by a counteracting shift of an ethylenic band or changed by mixing with an ethylenic mode in the shifted case. The positive band at 1557 cm^{-1} in Figure 2c indicates the presence of ethylenic bands in the lower region which are sensitive to this label. The additional shift of the band at 1578 cm^{-1} to 1570 cm^{-1} in the spectrum of 15- ^{13}C -labelled compounds in $^2\text{H}_2\text{O}$ (Gerwert *et al.*, 1985b) supports the assignment of the band at 1578 cm^{-1} , at least partially, to the shifted K C=N band. However, the subtraction indicates clearly that the K C=N stretching vibration has to be assigned to a band at about 1613 cm^{-1} and not to the band at 1609 cm^{-1} which was assigned by Rothschild *et al.* (1984) to the K C=N band. The latter band is still present in the spectrum of the 15- ^{13}C -labelled compound.

C-C-stretching and CCH-bending vibrations are located in the fingerprint region between 1400 cm^{-1} and 1100 cm^{-1} . Because their kinetic coupling depends on the configuration, this region is indicative of the isomerization state of the retinal. Figure 3 shows the BR-M difference spectra in the fingerprint region. The band at 1202 cm^{-1} (Figure 3a) is shifted to 1195 cm^{-1} by 15- ^{13}C -labelling, to 1193 cm^{-1} by 14- ^{13}C -labelling and to 1179 cm^{-1} by 14-15-di- ^{13}C -labelling (Figure 3b-d). Therefore, we assign this band to the $\text{C}_{14}-\text{C}_{15}$ stretching vibration of BR. The band at 1167 cm^{-1} , shifted by 10-11-di- ^{13}C -labelling to 1150 cm^{-1} (Figure 3c), is assigned to the $\text{C}_{10}-\text{C}_{11}$ stretching vibration of BR. Both assignments are in agreement with Resonance Raman data (Smith *et al.*, 1985a).

In the fingerprint region the BR-K difference spectrum (Figure 4Aa) shows additionally, as compared with the BR-M difference spectrum, a strong positive band at 1195 cm^{-1} with shoulder at 1187 cm^{-1} . The band with shoulder is shifted in the difference spectra to 1192 cm^{-1} and 1176 cm^{-1} by 14-15-di- ^{13}C -

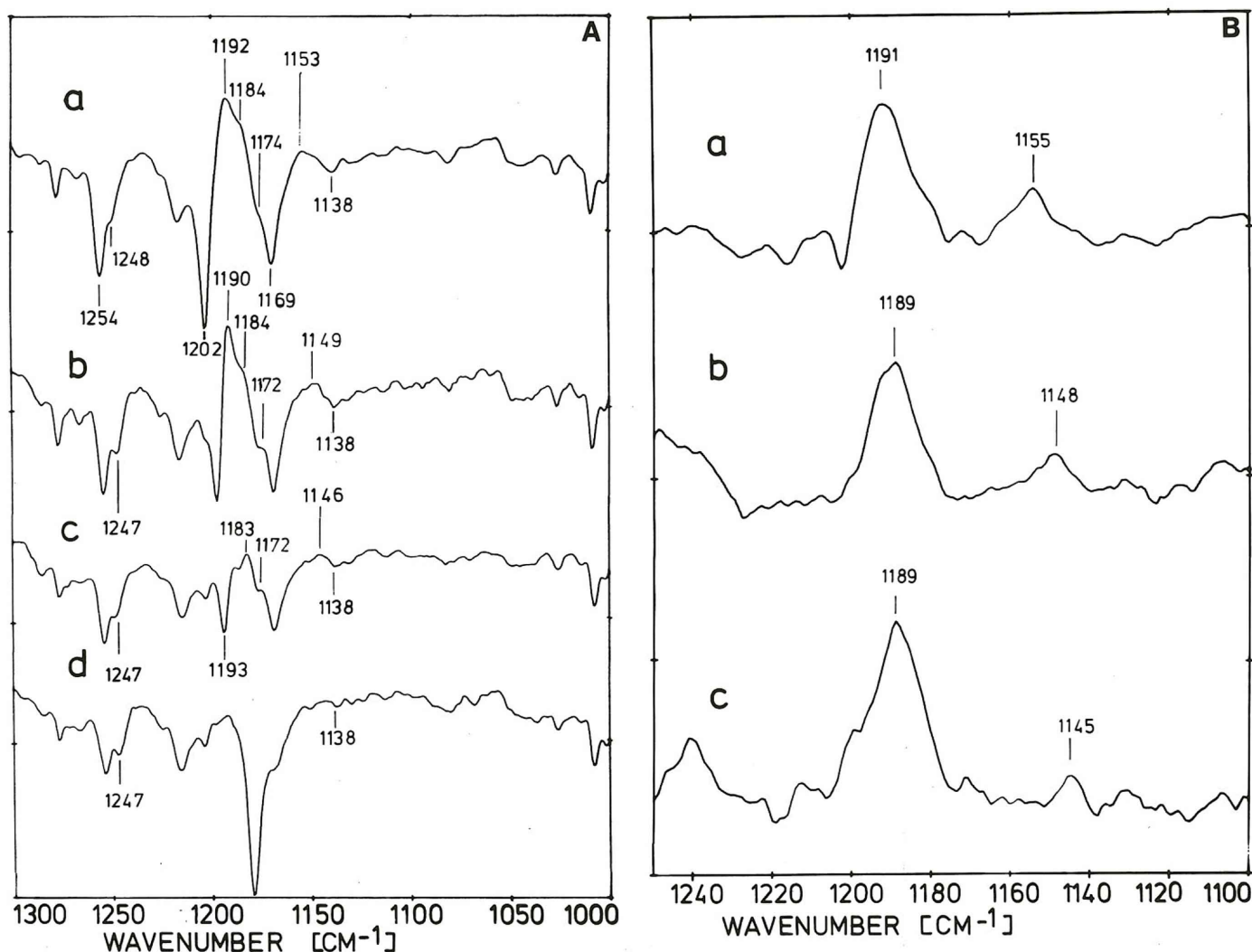


Fig. 5. (A) BR-L difference spectra in the fingerprint region for unlabelled (a), $^{15}\text{-}^{13}\text{C}$ -labelled (b), $^{14}\text{-}^{13}\text{C}$ -labelled (c) and $^{14}\text{-}^{15}\text{-di-}^{13}\text{C}$ -labelled (d) compounds in H_2O . (B) The L bands, obtained by subtracting the BR bands as described in Materials and methods, are shown for unlabelled (a), $^{15}\text{-}^{13}\text{C}$ -labelled (b) and $^{14}\text{-}^{13}\text{C}$ -labelled (c) compounds.

labelling and to 1184 cm^{-1} and 1166 cm^{-1} by $^{10}\text{-}^{11}\text{-di-}^{13}\text{C}$ -labelling. Therefore we assigned the K bands to a mixing of mainly $\text{C}_{10}\text{-C}_{11}$ and $\text{C}_{14}\text{-C}_{15}$ stretching vibrations (Gerwert *et al.*, 1985a, b). Because in the BR-K difference spectra the BR bands influence the position of the K bands, we subtracted the BR-M difference spectra, obtaining thereby essentially K bands (Figure 4B) as described in Materials and methods. Due to $^{10}\text{-}^{11}\text{-di-}^{13}\text{C}$ -labelling, the band at 1195 cm^{-1} loses intensity, the band at about 1187 cm^{-1} gains intensity and a new band appears at 1165 cm^{-1} . We can largely exclude that part of the band at 1195 cm^{-1} is shifted to 1165 cm^{-1} , because such a large shift due to ^{13}C -labelling of a stretching vibration is not observed in any other intermediate. Therefore, it is most likely that part of the band at 1195 cm^{-1} is shifted to 1184 cm^{-1} and at least part of the band at 1187 cm^{-1} is shifted to 1165 cm^{-1} , and that by $^{14}\text{-}^{13}\text{C}$ -labelling (Figure 4Bc) part of the band at 1195 cm^{-1} is shifted to 1185 cm^{-1} and at least part of the band at 1187 cm^{-1} is shifted to 1171 cm^{-1} . This assignment is supported by the further shifts of these bands to 1177 cm^{-1} and 1168 cm^{-1} by $^{14}\text{-}^{15}\text{-di-}^{13}\text{C}$ -labelling. The subtractions confirm our earlier assignment of the bands at 1195 cm^{-1} and 1187 cm^{-1} to mainly a mixing of $\text{C}_{14}\text{-C}_{15}$ and $\text{C}_{10}\text{-C}_{11}$ stretching vibrations.

The assignment of the $\text{C}_{14}\text{-C}_{15}$ stretching vibration in L is difficult because the intensity of most chromophore bands in L relative to BR is reduced. The main effect in the BR-L difference spectra (Figure 5A) is caused by the shift of the BR $\text{C}_{14}\text{-C}_{15}$ -stretching vibration at 1202 cm^{-1} under the L band at 1192 cm^{-1} whose intensity is thereby decreased. Regarding L, the band at 1153 cm^{-1} is most sensitive to the labels at the $\text{C}_{14}\text{-C}_{15}$ position. The band is shifted to 1149 cm^{-1} (Figure 5Ab), 1146 cm^{-1} (Figure 5Ac) and 1138 cm^{-1} (Figure 5Ad). The latter position can be deduced from the disappearance of the negative band at 1138 cm^{-1} , present in other spectra. Subtracting again the BR-M difference spectra (Figure 5B), two main bands at 1191 cm^{-1} and 1155 cm^{-1} were obtained. In the difference spectra, the position of the band at 1155 cm^{-1} is shifted to 1153 cm^{-1} due to the influence of the negative band at 1169 cm^{-1} . By $^{15}\text{-}^{13}\text{C}$ -labelling the band at 1155 cm^{-1} is shifted to 1148 cm^{-1} (Figure 5Bb) and by $^{14}\text{-}^{13}\text{C}$ -labelling to 1145 cm^{-1} (Figure 5Bc). Because the shifts are the same as observed in BR, and no other band is so sensitive to the labels as the band at 1155 cm^{-1} , we assign the band at 1155 cm^{-1} to the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration of L. This assignment is in contradiction to that of Smith *et al.* (1984), who assigned a shoulder at 1172

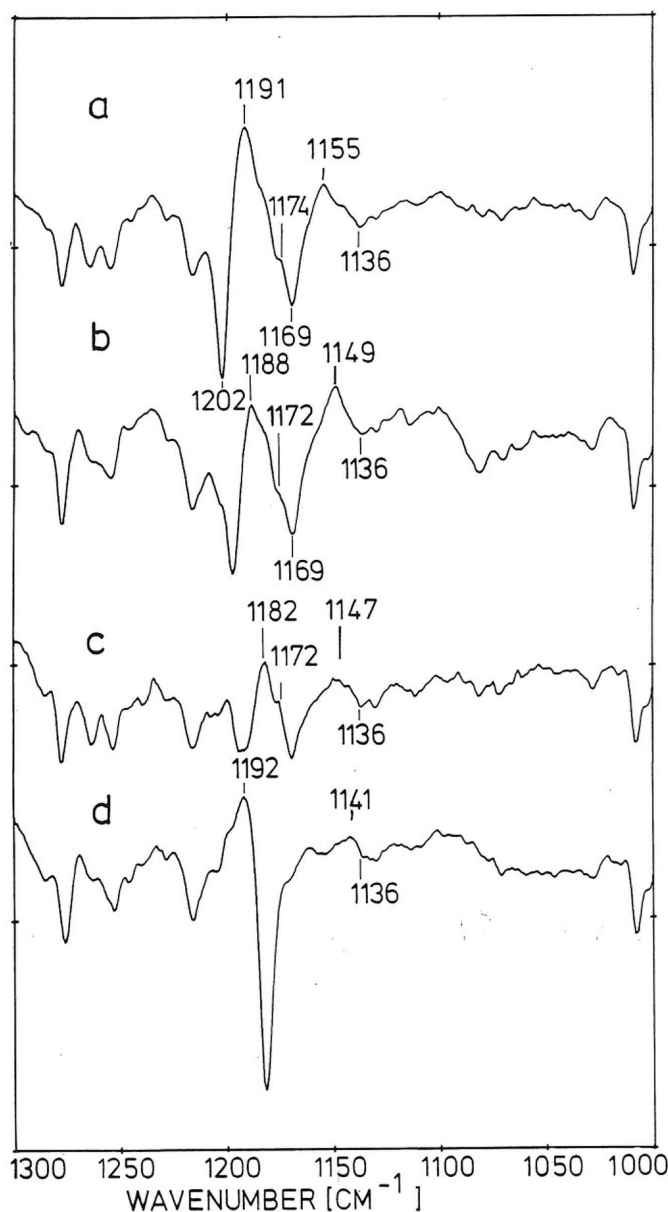


Fig. 6. BR-L difference spectra in the fingerprint region for unlabelled (a), $15\text{-}^{13}\text{C}$ -labelled (b), $14\text{-}^{13}\text{C}$ -labelled (c) and $14\text{-}15\text{-di-}^{13}\text{C}$ -labelled (d) compounds in $^2\text{H}_2\text{O}$.

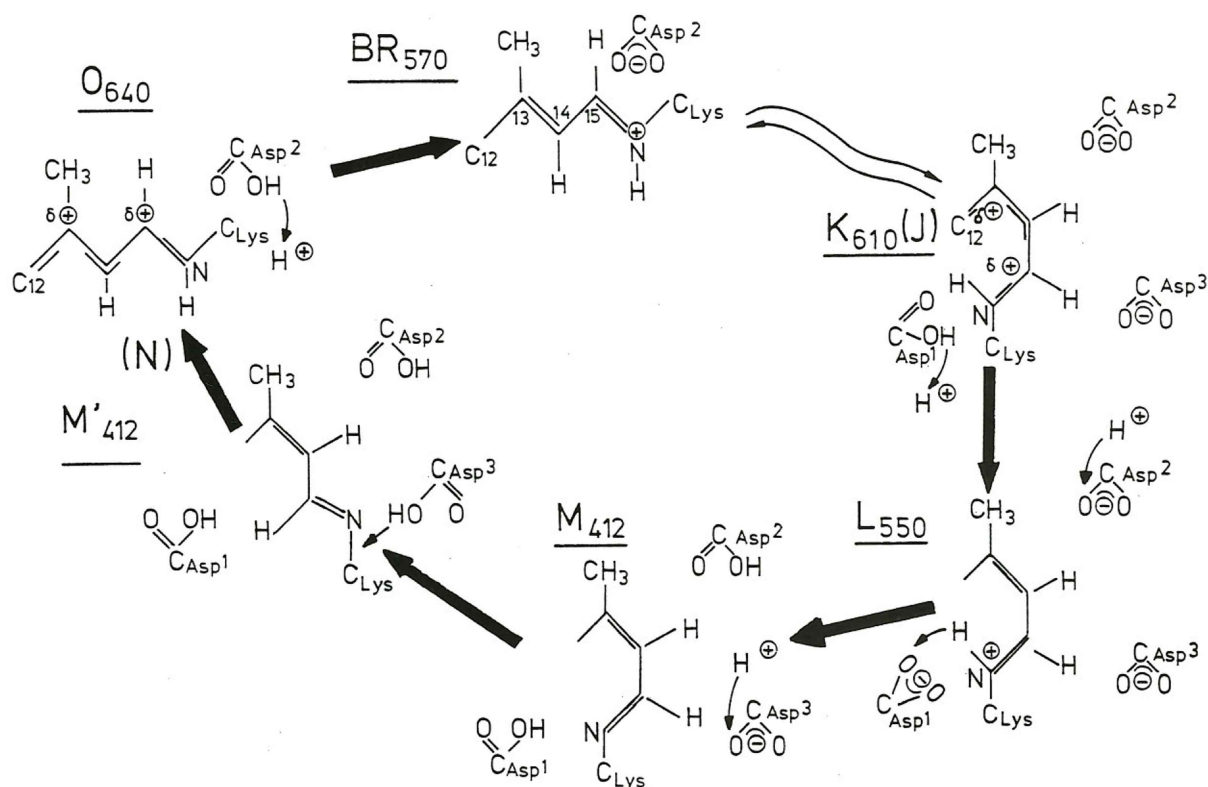
cm^{-1} in the Resonance Raman spectra to this mode. In the i.r. difference spectra the shoulder at 1172 cm^{-1} is only two wavenumbers shifted by $15\text{-}^{13}\text{C}$ and $14\text{-}^{13}\text{C}$ -labelling, in contrast to the band at 1155 cm^{-1} . The minor band at about 1170 cm^{-1} observed in the subtraction is too small for a reliable assignment. Also this band is still present in the spectrum of $14\text{-}^{13}\text{C}$ -labelled compounds (Figure 5Bc). Because of the importance of the assignment of the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration in L, we also measured the same labelled compounds in D_2O . In Figure 6 the same shifts of the band at 1155 cm^{-1} are observed as in H_2O . By $15\text{-}^{13}\text{C}$ -labelling, the band is shifted to 1149 cm^{-1} , by $14\text{-}^{13}\text{C}$ -labelling to 1147 cm^{-1} and by $14\text{-}15\text{-di-}^{13}\text{C}$ -labelling to about 1140 cm^{-1} . The subtraction and the difference spectra in $^2\text{H}_2\text{O}$ confirm our assignment of the band at 1155 cm^{-1} to the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration of L. The discrepancy between i.r. and RR data could be caused by a very low RR intensity of the

$\text{C}_{14}\text{-C}_{15}$ -stretching vibration in L. The RR intensities are also influenced by electronic excited states. Indeed, the intensity of ethylenic bands and HOOPs in L are reduced to a fifth of the BR intensity, which points towards a modified interaction of the protonated Schiff base with the protein-environment (Gerwert, 1985).

Discussion

Based on the above results, we can now draw conclusions regarding $\text{C}_{14}\text{-C}_{15}$ isomerization and changes of the protein environment at the terminal part of the chromophore. In retinal PSB model compounds the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration is shifted down by all-*trans* to 13-*cis* isomerization from 1191 cm^{-1} (Smith *et al.*, 1985) to 1174 cm^{-1} . For a 13-*cis* retinal PSB model compound the band at 1174 cm^{-1} had been assigned to $\text{C}_{14}\text{-C}_{15}$ -stretching vibration mode (Braiman and Mathies, 1980; Mathies *et al.*, 1986). Since the main ethylenic modes are at similar frequencies (1565 cm^{-1} in all-*trans*, 1572 cm^{-1} in 13-*cis*), the electronic delocalization seems similar in both configurations. Thus, the downshift is mainly caused by geometric changes (kinetic effects), which lead to different coupling of the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration with bending vibrations. This conclusion is supported by vibrational analysis of retinals, which showed that all-*trans* to 13-*cis* isomerization causes a downshift of about 20 wave-numbers due to kinetic effects (Curry *et al.*, 1984).

But, comparing the frequencies of the $\text{C}_{14}\text{-C}_{15}$ -stretching vibrations in BR and L, a downshift of 44 cm^{-1} is observed. The larger downshift can be caused either by drastically decreased force constant of the $\text{C}_{14}\text{-C}_{15}$ bond in L as compared to BR or by an additional isomerization around the $\text{C}_{14}\text{-C}_{15}$ bond. In model compounds such as butadien and acrolein, isomerization around the central single bond shifts the stretching vibration frequency down (Panchenko *et al.*, 1976; Bock *et al.*, 1980). For retinal PSB an empirical normal mode calculation of the terminal fragment predicted a downshift of 70 cm^{-1} by an isomerization around the $\text{C}_{14}\text{-C}_{15}$ bond, mainly due to kinetic effects (Smith *et al.*, 1986). The lowest feasible frequency for the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration caused by electronic effects, assuming a 13-*cis* configuration in L, is given by the 13-*cis* retinal PSB model compound. The lower frequency of the ethylenic modes in L at 1540 cm^{-1} and 1550 cm^{-1} (Stockburger *et al.*, 1979), as compared with those of the model compound at 1572 cm^{-1} , shows that the π -electrons of the retinal chain are less delocalized in the model compound than in L. Also the $\text{C}=\text{N}$ band is about 20 cm^{-1} lower in L (Argade and Rothschild, 1983) than in the model compound. The similar downshift due to $15\text{-}^2\text{H}$ -labelling indicates similar coupling to the vibrations of adjacent bonds (Gerwert, 1985). Thus, the $\text{C}=\text{N}$ bond order also seems lower in L than in the model compound. For these reasons the $\text{C}_{14}\text{-C}_{15}$ bond order can be expected to be lower in the model compound than in L and hence the lowest value for the stretching vibration in L for a 13-*cis* configuration should be 1174 cm^{-1} . The frequency of the $\text{C}_{14}\text{-C}_{15}$ -stretching vibration at 1155 cm^{-1} in L indicates, therefore, an isomerization around the $\text{C}_{14}\text{-C}_{15}$ bond, since this causes an additional downshift due to kinetic effects. The observed downshift is less than that expected from the empirical normal mode calculates. But this calculation accounted mainly for kinetic effects which can be counteracted by electronic changes. Quantum-chemical calculations which accounted for both effects demonstrated that depending on the distance of the counter-ion from the Schiff base, the downshift can vary between 10 and 70 cm^{-1} in a 14-*s-cis* configuration (P. Tavan and K. Schulten, manuscript in preparation). The calculations show-



Model describing the transduction of light-energy into proton transfers in BR. Light-induced 13-*cis*, 14-*s-cis* isomerization exposes the Schiff base in K to a less polar environment, leading to a migration of the positive charge into the retinal chain. The altered charge distribution cannot be compensated by the protein at this time scale. But unshielded charges inside a protein are energetically unfavourable. In order to reduce the energy, asp(1), which is near the Schiff base, is de-protonated in the K to L transition. The hindered 14-*s-cis* configuration reduces the pK of the Schiff base and leads to proton transfer from the nitrogen to asp(1) in the L to M transition. Concomitant asp(2) is also protonated. Separate re-isomerization around the C₁₄-C₁₅ bond transposes the Schiff base in the M to M' transition near asp(3) which donates a proton. After re-protonation (N), re-isomerization around the C₁₃=C₁₄ double bond can occur, because the isomerization barriers are decreased. A vectorial transport can be explained if asp(1) and asp(3) are accessible for protons only from different sides of the membrane.

ed also that in this configuration the C₁₄-C₁₅-stretching vibration frequency is more sensitive to the position of the counter-ion than in the all-*trans* configuration.

Because isomerization around the C₁₄-C₁₅ bond in the K to L transition is unlikely (Tavan *et al.*, 1985), also a 13-*cis*, 14-*s-cis* configuration must be assumed for K. The downshift of only 10 wave-numbers for the C₁₄-C₁₅-stretching vibration in K can be caused by counteracting electronic effects. The downshift of the C=C-stretching vibration in K to 1517 cm⁻¹ indicates a larger electron delocalization in the retinal chain as compared to L. Also the C=N stretching vibration at 1613 cm⁻¹ is unusually low. But the different isotopic shifts due to 15-¹³C- and N-²H-labelling in K as compared with L indicate a change of the coupling to the vibrations of adjacent bonds which also influence the frequency (Kakitani *et al.*, 1983). In order to account for the different coupling we performed a vibrational analysis of the Schiff base region for BR, K and L as described in Materials and methods. The analysis showed that, in comparison with BR, the C=N bond order in K decreases by about 0.1. Therefore it must be concluded that in K the chromophore is transformed towards a retinyl cation. In agreement with the experimentally observed value, quantum-chemical calculations (K. Schulten and P. Tavan, manuscript in preparation) predicted only a difference of about 10 wave-numbers between the frequencies of the C₁₄-C₁₅-stretching vibration for the all-*trans* configuration with counter-ion and the 13-*cis*, 14-*s-cis* geometry without counter-ion. Thus, the small observed downshift is not

in contradiction to a 13-*cis*, 14-*s-cis* configuration in K.

From the decrease of the C=N bond order in K it must be concluded that light-induced isomerization exposes the terminal part of the chromophore in K to a protein environment which is no longer able to stabilize the positive charge at the Schiff base. It was shown that for an isolated retinal PSB the positive charge is distributed into the retinal chain (Honig, 1978). This lowers the double-bond orders and increases those of the single bonds. By adding a counter-ion near the Schiff base the positive charge is attracted and the charges are reversed.

For the C=N bond order in L we obtained an increase of about 0.14 relative to K. This indicates that the positive charge is again fixed at the terminal part of the protein environment.

Taking into account our earlier results the model shown in Figure 7 can be derived. Light-induced isomerization exposes the Schiff base to a less polar environment and the positive charge is distributed into the retinal chain. Since the dielectric relaxation time of the protein interior is long as compared to the transition time, the charges are unshielded and the protein reaches an energetically higher state (Warshel and Russell, 1984). Because the terminal part of the chromophore is then positioned near asp(1), the protein is able to stabilize the charges by de-protonation of asp(1) in the K to L transition. The hindered 14-*s-cis* configuration reduces the pK of the Schiff base nitrogen (Schulten and Tavan, 1978) and in the L to M transition the proton can be transferred to asp(1). Concomitant asp(2) is protonated, since otherwise an isolated charge would be present inside the protein.

Re-protonation can be obtained by an isomerization around the C₁₄–C₁₅ bond in the M to M' transition (Schulten *et al.*, 1984). Thereby the Schiff base is transposed near asp(3) which can donate a proton to the Schiff base. Asp(3) undergoes protonation changes at this time scale (Siebert *et al.*, 1982; Engelhard *et al.*, 1985). After re-protonation re-isomerization to all-*trans* can occur. If asp(1) and asp(3) are only accessible for protons from different sides of the membrane, this model can also explain a vectorial transport. By the proposed model the transduction of light energy into proton transfers can be described.

Materials and methods

Purple membranes were isolated, bleached and regenerated by labelled retinals as described (Oesterhelt and Stoekenius, 1974; Kunaga and Ebrey, 1978). The isotopically labelled retinals were synthesized as described by Lugtenburg (1985) and Pardoën *et al.* (1984). Difference spectra were obtained as described by Engelhard *et al.* (1985) and Siebert and Mäntele (1983).

Because the BR-M difference spectra reflect, regarding chromophore bands, mostly BR bands (a protonated retinyl Schiff base absorbs much stronger in the i.r. than an unprotonated) the BR chromophore bands can be assigned in these difference spectra. As an appropriate approximation we assigned the band which is most sensitive to a specific labelling to the corresponding internal mode.

To assign reliably the C=N-stretching vibrations, the spectra of the unlabelled and 15-¹³C-labelled compounds were subtracted. The constants for the subtractions in the Schiff base region were obtained by linear regression of the distances between the subtracted spectra between 1800 cm⁻¹ and 800 cm⁻¹, using the software of the Bruker FTIR instrument. In Figure 1 three subtractions between different spectra were added, and in Figure 2, five. In order to elucidate in the fingerprint region the influence of BR bands, we subtracted the BR-M difference spectra which shows in this region only BR bands. The subtraction constants were determined by compensating the BR bands at 1255 cm⁻¹, 1215 cm⁻¹, 1202 cm⁻¹, 1167 cm⁻¹ and 1008 cm⁻¹. Variations of the constants around the selected values did not essentially alter the results but produced additional bands, either positive or negative, caused by the BR-bands.

Vibrational analyses were performed with the normal mode program developed by Curry (1983) using the Wilson FG method (Wilson *et al.*, 1955). Because the C=N-stretching vibration is a good group frequency, as shown by vibrational analysis of the PSB retinal (Smith *et al.*, 1985), it is an appropriate approximation to calculate the Schiff base region only. Starting with the force field as derived by Smith *et al.* (1984), we fitted the frequencies obtained for BR, K and L for the unlabelled and 15-¹³C-labelled compounds in H₂O and ²H₂O by varying the diagonal force constants for stretching and bending vibrations. The analysis accounted for the different coupling of the C=N-stretching vibration with the bending and stretching vibrations of adjacent bonds which also influence the C=N frequency (Kakitani *et al.*, 1983). Bond orders were obtained using the relationship between diagonal stretching force constants (F_{ij}) and bond orders (P_{ij}) $P_{ij} = 6.4 F_{ij} + 4.4$ for linear polyenes (Gavin and Rice, 1971).

Acknowledgements

We would like to express our special gratitude to J. Lugtenburg and his co-workers C. Winkel and J.A. Pardoën for providing us with the excellent isotopically labelled retinals. We are grateful to R. Mathies and his co-worker A. Myers for introducing one of us (K.G.) to the normal mode program and providing us with it. We thank P. Tavan for stimulating discussions and for having made available to us the results of the calculations prior to publication. Technical assistance by G. Heppeler and valuable discussions with W. Kreutz are gratefully acknowledged. We thank T. Ackermann for granting measuring time on the FTIR-spectrophotometer. This work was supported by the Deutsche Forschungsgemeinschaft SFB 60 D-10 and A-4.

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Received on 9 December 1985; revised on 27 January 1986