Biophys. Struct. Mech. 6, 147–164 (1980)

Biophysics of Structure and Mechanism

Investigations of the Rhodopsin/Meta I and Rhodopsin/Meta II Transitions of Bovine Rod Outer Segments by Means of Kinetic Infrared Spectroscopy

F. Siebert and W. Mäntele

Institut für Biophysik und Strahlenbiologie der Universität Freiburg im Breisgau, Albertstraße 23, D-7800 Freiburg im Breisgau, Federal Republic of Germany

Abstract. We have applied our recently developed technique of flash induced kinetic infrared spectroscopy to the rhodopsin/Meta I and rhodopsin/Meta II transitions. Features of the infrared spectrum reflecting the C=C-vibration and the isomeric form of the chromophore are in agreement with resonant Raman experiments. Different results are obtained for the C=N-vibration of the Schiff base retinal opsin link. They are interpreted in terms of a Schiff base protonated via an hydrogen bond. A proton transfer in the excited state is suggested to explain the deviating results. In addition we have obtained spectral changes which cannot be attributed to molecular changes in the chromophore. We assume that these spectral features reflect molecular events in the protein part of rhodopsin.

Key words: Rhodopsin – Protein retinal linkage – Opsin transitions – Kinetic infrared spectroscopy.

Introduction

Rhodopsin, the visual pigment in photoreceptor cells responsible for the transduction of a light stimulus into a neural response, is composed of 11-*cis*-retinal bound to a glycoprotein called opsin. Upon absorption of a photon, it undergoes a series of thermal transitions whose intermediates are characterized by their broad absorption bands in the visible or ultraviolet spectral range. The spectra were obtained either by flash photolysis experiments (for a review see Abrahamson and Wiesenfeld 1972), or by spectroscopy at reduced temperatures to freeze the intermediates (for a review see Yoshizawa 1972). The endproduct of the reaction sequence is all-*trans*-retinal detached from opsin. In recent years additional information about the state of retinal in rhodopsin was obtained by resonance Raman experiments (Lewis et al. 1973; Oseroff and Callender 1974; Mathies et al. 1976). One of the important results obtained by this method was the confirmation that the Schiff base retinylidene link is protonated (Oseroff and Callender 1974). The protonated Schiff base, however, has recently been questioned by experimental results (Peters et al. 1977; Abrahamson et al. 1977) and on the basis of the-

0340-1057/80/0006/0147/$ 03.60

oretical arguments (Favrot et al. 1979; Hárosi et al. 1978). A protonation via an hydrogen bond was suggested and a proton transfer proposed as the first molecular event after the absorption of the photon. To explain the results from resonance Raman experiments it was argued that molecular rearrangements occurring in the excited state from which the photon in resonance Raman experiments is reemitted, could influence the observed spectrum.

In order to gain information about this problem by a method which does not involve the excited electronic state, we decided to apply our recently developed method of flash induced kinetic infrared spectroscopy (Siebert et al. 1980) to the photochemistry of rhodopsin. In addition to the problem of the retinal opsin linkage, the kinetic infrared spectroscopy applied to rhodopsin may reveal changes in the protein part playing a role in the rhodopsin function. Hitherto it was not possible to unequivocally identify such molecular changes (for a recent discussion see Rafferty et al. 1977; Rafferty 1979). They can however, inferred from chemical investigations (De Grip et al. 1975; Fung and Hubbel 1978) and from the fact that a fast proton uptake is observed after the rhodopsin/Meta I transition (Falk and Fatt 1966). The selectivity of resonance Raman scattering, its great advantage in other respects, will not allow to observe directly molecular changes not belonging to the chromophore. In our previous paper (Siebert et al. 1980), we demonstrated that it is possible to observe in the infrared small spectral changes in the millisecond time scale even in the presence of the background absorption of such a large macromolecule as rhodopsin. In this paper it will be shown that it is possible to assign these spectral changes to specific molecular events. It will be further shown, that for many spectral features agreement is observed with resonance Raman measurements, but that for vibrations representing the state of the Schiff base linkage deviating results are obtained. In addition, evidence will be presented for molecular changes not belonging to the chromophore and manifesting with time courses different from the reaction times of the chromophoric transitions.

Materials and Methods

The apparatus developed by us to obtain flash induced kinetic infrared spectra was described previously (Siebert et al. 1980). It resembles a conventional flash photolysis instrument, i.e., flash induced transmission changes of infrared radiation passing through the sample are measured. The time resolution is about 5 ms for relative transmission changes of 10^{-3}. If several signals are summed up in a signal averager the time resolution can be improved correspondingly.

Rod outer segments (ROS) from bovine retinae were prepared as previously described (Siebert et al. 1977). They are characterized by an absorbance ratio A_{280}/A_{498} of 2.3 to 2.4, measured in 1% sodiumdeoxicholate. For the measurements of the rhodopsin/Meta I and rhodopsin/Meta II difference spectra in the infrared, three types of samples were prepared: 1. As our instrument is not yet fast enough to resolve the Lumi/Meta I transition, the decay of Meta I was stopped by drying the sample (Wald et al. 1950). In this case, ROS were centrifuged from a suspension on a CaF_2-window. The supernatant was soaked with a syringe and the sample dried by a water jet pump. The sample was sealed from the atmosphere with a second CaF_2-window and an O-ring. This type of sample is called "dried film sample". 2. For measurements of the

rhodopsin/Meta II transition with the infrared beam near the water absorption band at 1,650 cm^{-1}, the dried films were rehydrated for 1 h in a closed box under the presence of a saturated $K_2Cr_2O_7$-solution. This sample was also sealed from the atmosphere by a second CaF_2-window and an O-ring. It is called "hydrated film sample". 3. For the measurements of the rhodopsin/Meta II transition outside the water absorption band an ROS-suspension was sedimented in a small polyethylene tube (4 mm ∅), the tube was frozen in liquid nitrogen and cut slightly below the interface between sediment and supernatant. The sediment was pressed out on a CaF_2-window and squeezed by a second window to a spacing of 12 μm.

Deuterium exchange was performed by washing the ROS twice in D_2O-buffer and by subsequent equilibration of the suspension for 12 h at 4° C. The deuterated dried film samples were prepared from a deuterated suspension. For drying the sample a tube filled with silica gel was placed between the sample and the water jet pump. By this method a quick drying without H_2O-reexchange was achieved. The degree of deuterium exchange was monitored by measuring the decrease of the amide II-band (Osborne and Nabedryk-Viala 1977).

Model compounds, i.e., all-*trans*-N-retinylidene-n-butylamine (all-*trans*-NRBA), the 11-*cis* isomer (11-*cis*-NRBA), their chloride salts (-NRBA-HCl) and the perchlorate of all-*trans*-NRBA (all-*trans*-NRBA-$HClO_4$) were prepared under dim red light according to the method of Blatz et al. (1972). Also the solvents were purified and dried as described in this paper. Infrared spectra, taken with the model PE 180 infrared spectrophotometer from Perkin-Elmer were obtained in dichloromethane, chloroform and methanol. We used a cuvette with a spacing of 50 μm equipped with two CaF_2-windows. KBr windows could not be used, since the bands were strongly broadened, probably due to partial anion-exchange. All-trans retinal was from Fluka, Neu-Ulm, and supposed to have a purity of 95%. This was verified by comparing the infrared spectrum with a published spectrum (Robeson et al. 1955). 11-*cis*-retinal was a gift from Hoffmann-La Roche, Basel. Its purity was estimated to at least 90%, from ultraviolet and infrared spectra (Robeson et al. 1955).

To correct for solvent evaporation during the preparation of the infrared samples of the model compounds, the integrated absorbance between 3,200 cm^{-1} and 2,700 cm^{-1} (C-H-, $C-H_2$-, and $C-H_3$ stretching vibrations) was used to normalize the spectra.

Results

The infrared survey spectra of the three types of samples used for the measuring of the rhodopsin/Meta I and rhodopsin/Meta II difference spectra are shown in Fig. 1. The transmission around 1,650 cm^{-1} is very low, not only due to the water absorption band but also to the strong amide I-band of the proteins. The band around 1,230 cm^{-1}, probably the P=O-stretching vibration of the phospholipids shows the well known shift to higher wavenumbers upon removal of water (Chapman et al. 1967). The relative amount of water to ROS was determined by the ratio of the absorbance at 3,400 cm^{-1} (O-H-vibration of water) and at 1,740 cm^{-1} (C=O-vibration of phospholipids) (Chapman et al. 1966).

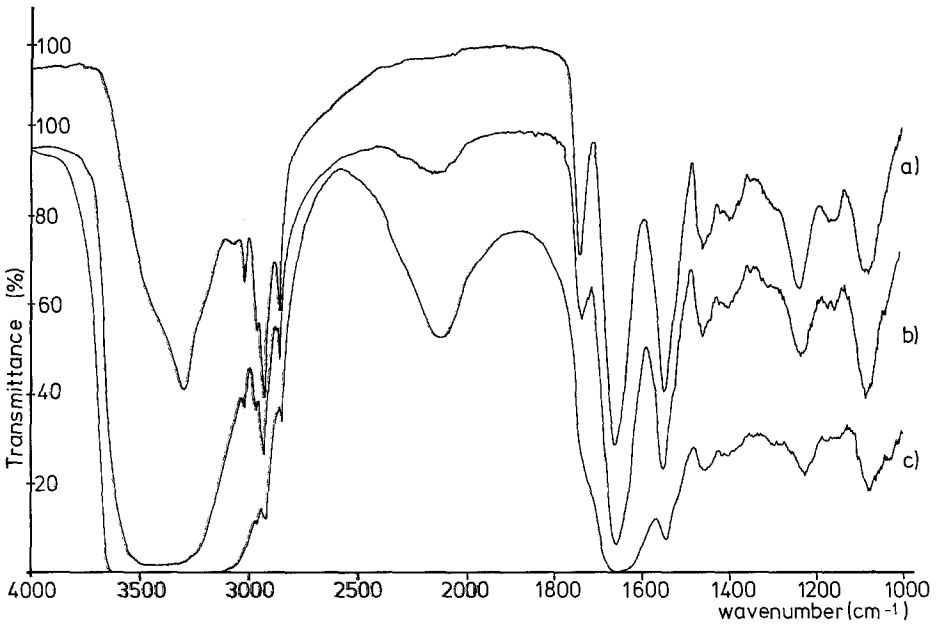

Fig. 1. Survey infrared spectra of the three types of bovine rod outer segment samples used in kinetic spectroscopy. **a** Dried film sample; **b** hydrated film sample; **c** aqueous squeezed sediment sample. Spectra were taken with a model PE 180 Infrared Spectrophotometer from Perkin-Elmer. Resolution varied between 3 cm^{-1} and 4 cm^{-1}. Spectrum a is shifted upward compared to spectra b and c

A typical signal of the rhodopsin/Meta II transition from an aqueous squeezed sediment sample observed at 1,195 cm^{-1} is shown in Fig. 2a. The sample was cooled to 15° C to better detect the rhodopsin/Meta I transition. Approximately 20% of the rhodopsin was bleached by the flash with wavelengths greater than 530 nm. Despite the electronic interference produced by the discharge of the flash, a negative change in transmission immediately after the flash can be seen. It corresponds to the rhodopsin/Meta I transition as confirmed by measurements with the dried film samples. The negative jump is followed by a slower positive signal. It has the same half time as the rhodopsin/Meta II transition observed at 380 nm. Preliminary measurements of the temperature dependence of the signal at 1,195 cm^{-1} reveal that the amplitude is in agreement with a temperature dependent Meta I/Meta II equilibrium, and that the enthalpy of activation is 32 $kcal/mol^{-1}$, in accordance with published results (Abrahamson and Wiesenfeld 1972).

In Fig. 2b we show a signal at 1,235 cm^{-1} measured under the same conditions. Since no slow component is observed, we conclude that the transmission at this wavenumber does not change during the Meta I/Meta II transition. This was verified by measuring the signals of two identical hydrated film samples at this wavenumber, of which one was completely dried to stop the Meta I/Meta II transition. The signals obtained for the two samples were undistinguishable within the limits of error. Thus, the signal at 1,235 cm^{-1} offers a convenient possibility to normalize the signals at other wavenumbers: The transmission changes induced by the second and third flash were measured at 1,235 cm^{-1}, and the signal amplitude ΔI_∞ (Fig. 2), extrapolated to its value

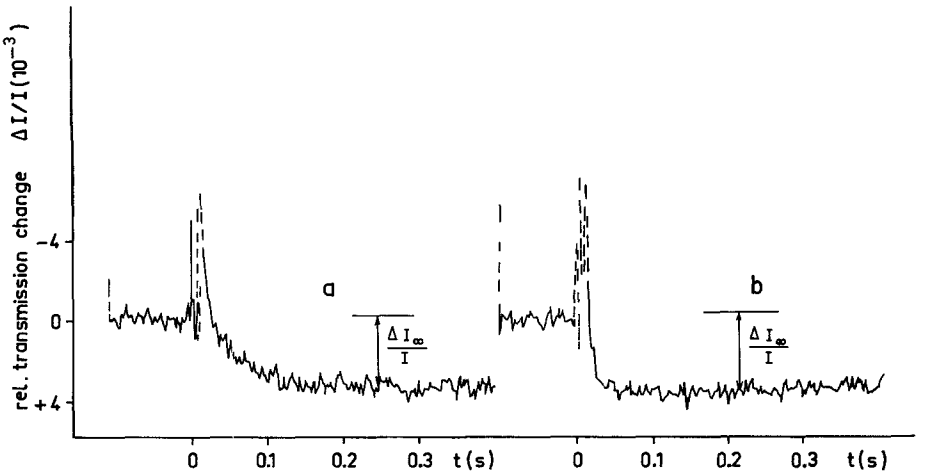

Fig. 2. Flash induced signals of an aqueous squeezed sediment sample of bovine ROS. **a** Measured at 1,195 cm^{-1}; **b** measured at 1,235 cm^{-1}. Approx. 20% of the rhodopsin was bleached by the flash from which light with wavelengths shorter than 530 nm was cut off by a filter. The same sample was used for both wavenumbers, the signal at 1,235 cm^{-1} was triggered by the second flash. Flash duration was approximately 1 ms. $\Delta I_\infty/I$ is the final relative transmission change

at the first flash. This value is a direct measure of the amount of rhodopsin bleached by the first flash independent of pH and temperature. This method allows the compensation of concentration differences occurring especially between the different types of samples. By measuring the spectral dependence of the thus normalized relative transmission change, $\Delta I_\infty/I$, the kinetic infrared difference spectra for the rhodopsin/Meta II and rhodopsin/Meta I transitions are obtained (Figs. 3 and 4, respectively). Each data point corresponds to a new sample. The terms Meta I and Meta II are only loosely used in this connection, since first they describe spectral properties of the chromophore only and secondly two isochromic forms of Meta I have been postulated recently (Uhl et al. 1978).

Model Compounds

The infrared spectra of all-*trans*-NRBA, all-*trans*-NRBA-HCl and 11-*cis*-NRBA-HCl in dichloromethane are shown in Fig. 5 (abbreviations see "Materials and Methods"). The position of the bands of the above in chloroform and methanol and of all-*trans*-NRBA-$HClO_4$ in chloroform are given in Tables 1 and 2. For comparison the data of resonance Raman measurements of model compounds and of rhodopsin are included (Mathies et al. 1976, 1977).

We first wish to make a few general comments on the infrared spectra of the model compounds. Upon formation of the Schiff base from the retinal isomers all bands in the region between 1,800 cm^{-1} and 1,000 cm^{-1} are reduced in intensity. In the case of all-*trans*-NRBA, bands at 1,338 cm^{-1} and between 1,140 cm^{-1} and 1,110 cm^{-1} disappear almost completely. In the case of the 11-*cis* isomer the situation is more complicated:

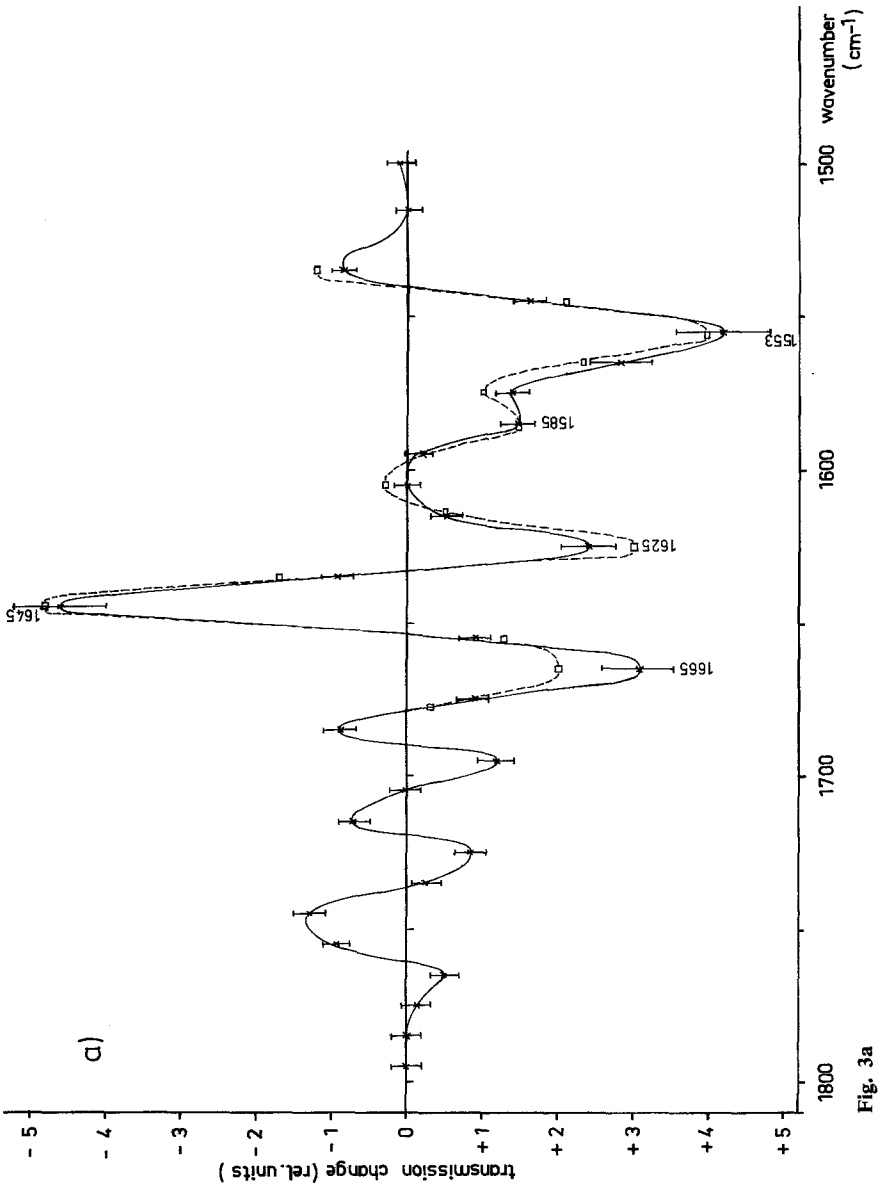

Fig. 3a

Fig. 3. Kinetic rhodopsin/Meta II difference spectrum. For each data point a new sample was used. Each flash bleached approximately 20% of the rhodopsin. **a** Spectral region between 1,800 cm^{-1} and 1,500 cm^{-1}. Hydrated film samples were used. Dashed line corresponds to the deuterated sample, in which case squeezed sediment samples were used; **b** spectral region between 1,500 cm^{-1} and 1,100 cm^{-1}. Squeezed sediment samples were used. Spectral resolution varied between 10 cm^{-1} and 6 cm^{-1}, depending on the infrared intensity at the detector. Ordinate scale unit corresponds to a relative transmission change of $\Delta I/I = 3 \times 10^{-3}$

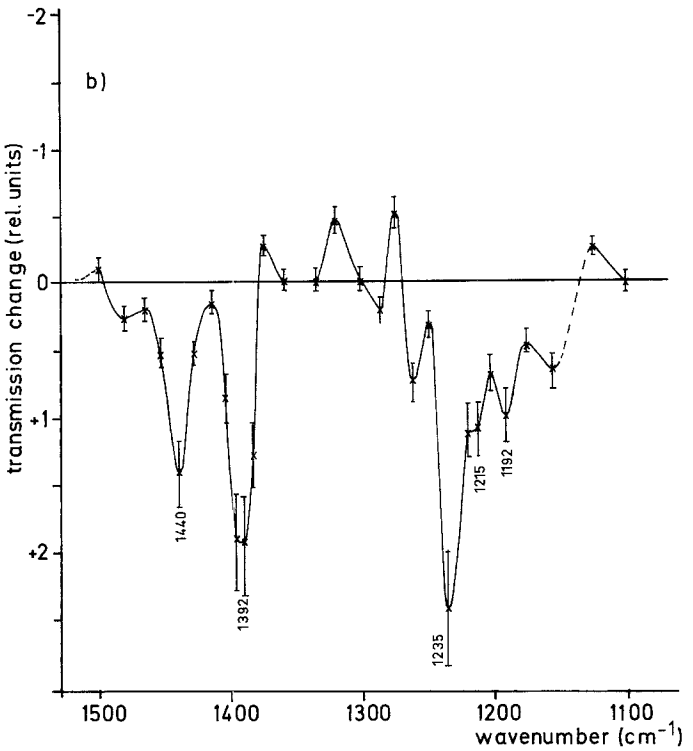

Fig. 3b

new bands are formed in addition at 1,220 cm^{-1} and 1,195 cm^{-1}. The intensity and position of these two bands depend strongly on the solvent. It might be that they reflect the equilibrium between the two highly twisted forms of the 11-*cis* isomer, the 12*s*-*cis*- and 12*s*-*trans*-forms (Sperling 1973; Rowan et al. 1974).

If the Schiff bases are protonated, all bands between 1,800 cm^{-1} and 1,100 cm^{-1} are increased. For the C=N-stretching vibration (1,670 cm^{-1} to 1,610 cm^{-1}), the C=C-stretching vibration (1,590 cm^{-1} to 1,520 cm^{-1}) and the fingerprint region (mostly C-C-stretching vibrations) factors from 5–9 are observed, whereas factors between 1 and 2 are observed for the C-H_2-deformation and the methyl-deformation vibrations (1,460 cm^{-1} to 1,350 cm^{-1}).

To compare the spectra of Fig. 5 with each other, they have to be multiplied with the factors of 1.0, 0.83, and 0.27 for all-*trans*-NRBA-HCl, 11-*cis*-NRBA-HCl and all-*trans*-NRBA, respectively.

The observed absorption power changes upon modification of retinal isomers to Schiff bases or protonated Schiff bases can be explained by changes of π-electron delocalization. Increased delocalization reduces bond alternations but increases charge alternation on the carbon atoms; therefore, the transition moments of vibrations involving these carbon atoms are increased. Due to the low absorption of NRBA, the difference spectrum between 11-*cis*-NRBA-HCl and all-*trans*-NRBA would essentially produce only positive transmission changes and approximately reproduce the 11-*cis*-NRBA-HCl spectrum.

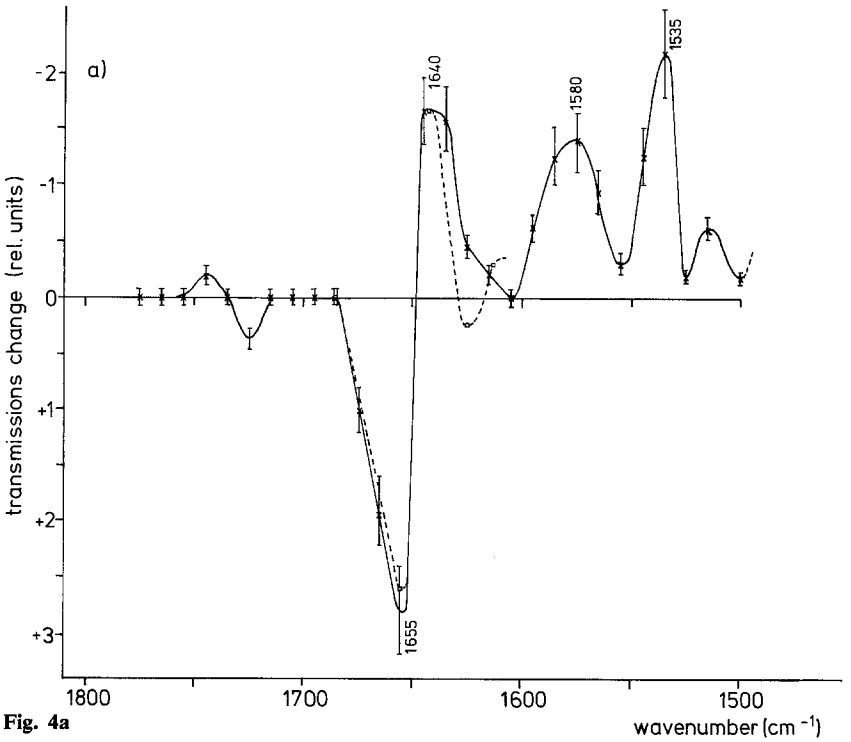

Fig. 4a

Fig. 4. Kinetic rhodopsin/Meta I difference spectrum. For each data point a new sample was used. Each flash bleached approximately 20% of the rhodopsin. **a** Spectral region between 1,800 cm^{-1} and 1,500 cm^{-1}. Dashed line corresponds to the deuterated sample; **b** spectral region between 1,500 cm^{-1} and 1,100 cm^{-1}. Spectral resolution varied between 10 cm^{-1} and 6 cm^{-1}, depending on the infrared intensity at the detector. Ordinate scale unit corresponds to a relative transmission change of $\Delta I/I = 3 \times 10^{-3}$

In addition to the decrease in absorbance the deprotonation of all-*trans*-NRBA-HCl shifts several bands: 1. the C=N-vibration is shifted from 1,652 cm^{-1} to 1,622 cm^{-1}; 2. the C=C-vibration is shifted from 1,552 cm^{-1} to 1,577 cm^{-1}; 3. the two bands in the fingerprint region 1,238 cm^{-1} and 1,191 cm^{-1} are shifted to 1,222 cm^{-1} and 1,172 cm^{-1}, respectively.

These shifts can also be explained by the decrease in charge delocalization, which results in larger bond alternation and therefore in an increase in C=C-frequency and a decrease in C–C-frequency.

In Fig. 5 it is evident that the fingerprint region and the C=C-vibration are dependent on the isomeric form of the retinal. More specifically, changing 11-*cis*-NRBA-HCl to all-*trans*-NRBA-HCl reduces a band at 1,232 cm^{-1} and shifts it to 1,238 cm^{-1} and increases a band at 1,180 cm^{-1} and shifts it to 1,192 cm^{-1}. The C=C-band is narrowed and shifted from 1,556 cm^{-1} to 1,552 cm^{-1}.

Comparing the resonance Raman data with the infrared data in methanol (Tables 1 and 2, the data in methanol are essentially identical with those in ethanol), one always observes deviations of band positions: bands in the fingerprint region are shifted to higher wavenumbers in resonance Raman experiments, whereas the C=C-vibration is shifted to lower wavenumbers. This effect is not easily explained.

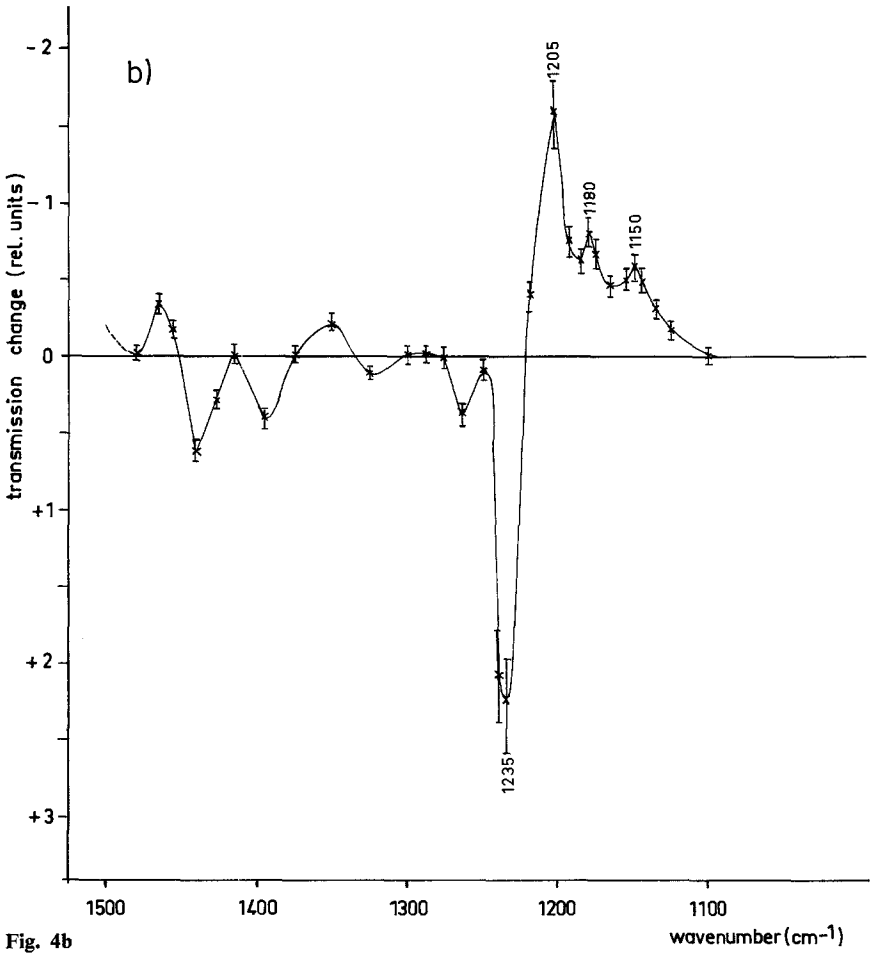

Fig. 4b

Discussion

Rhodopsin/Meta II Difference Spectrum

The kinetic infrared rhodopsin/Meta II difference spectrum, Fig. 3, shows, that for the most part of the spectrum only transmission increases are observed. It is generally accepted, that in Meta II the Schiff base of the retinal opsin link is deprotonated. As has been shown for the model substances, deprotonation causes a large decrease in absorption power for the spectral ranges 1,700 cm^{-1} to 1,500 cm^{-1} and 1,300 cm^{-1} to 1,100 cm^{-1}. The interpretation of the difference spectra in these spectral regions, therefore, is based on the assumption that they reflect the chromophore. All-*trans*-NRBA-$HClO_4$ can be taken as a model for Meta I, since the conformation of retinal in Meta I is supposed to be all-*trans* and the absorption maxima in the visible range, λ_{max}, are close to one another. The corresponding 11-*cis* isomer is not an equally good model for rhodopsin, as the λ_{max} differ by almost 30 nm. All-*trans*-NRBA can be used as a

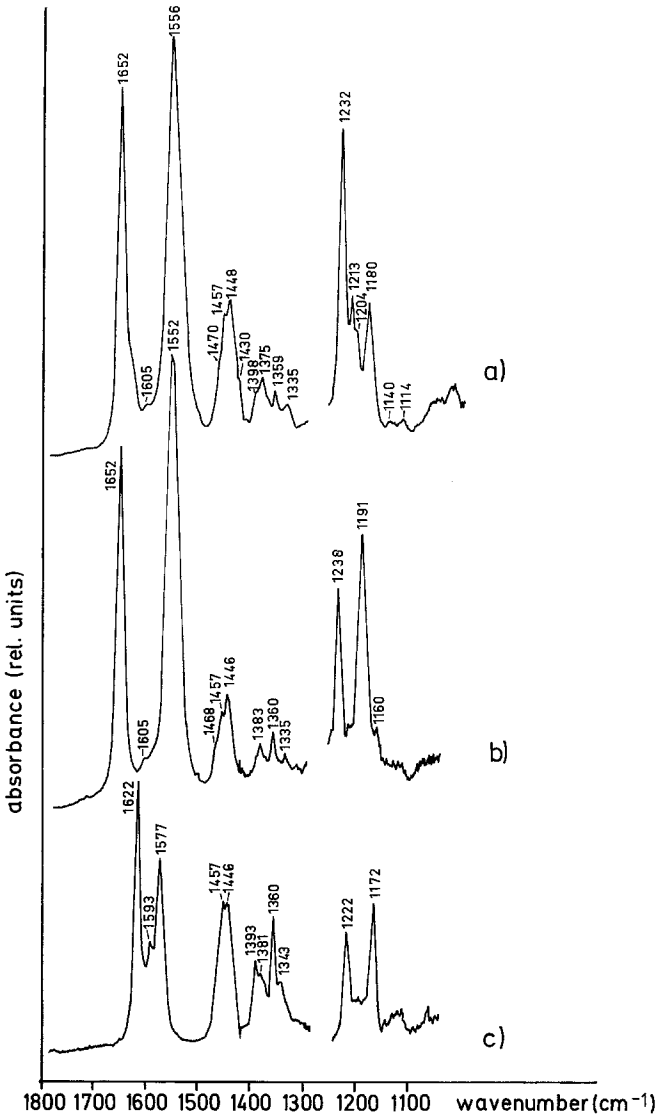

Fig. 5. Infrared spectra of model compounds. **a** 11-*cis*-NRBA-HCl; **b** all-*trans*-NRBA-HCl; **c** all-*trans*-NRBA. Infrared spectra were recorded with CH_2Cl_2 as solvent, concentration varied between 20 mg/ml and 50 mg/ml. A sample cell with two CaF_2-windows at a spacing of 50 μm was used. Resolution varied between 2 cm^{-1} and 3 cm^{-1}. Ordinates of the spectra of a, b, and c have to be multiplied by the factors of 0.83, 1.0, and 0.27, respectively. The gap between 1,300 cm^{-1} and 1,250 cm^{-1} is caused by the solvent absorption

model for Meta II. By using the model compounds for the interpretation of the difference spectra one has to take into account the effect of the protein on the retinal. Although relationships between λ_{max} of protonated Schiff bases and some of their vibration frequencies exist (Heyde et al. 1971; Rimai et al. 1973), these relationships are only approximate and show large deviations, depending on the solvent (e.g., vibrations

Table 1. Infrared- und resonance Raman-data of all *trans* model compounds (cm^{-1}). Measuring conditions were as in Fig. 5. Resonance Raman-data are from Mathies et al. 1977

Infrared				Resonance Raman
All-*trans*-NRBA-HCl in			All-*trans*-NRBA-$HClO_4$ in $CHCl_3$	NRBA-HCl in EtOH
CH_2Cl_2	$CHCl_3$	MeOH		
1,652	1,651	1,658	1,649	1,658
1,605	1,607	1,608	1,590	–
1,552	1,552	1,559	1,550	1,555
1,470	1,470	–[a]	1,470	–
1,457	1,456	–[a]	1,456	1,456
1,446	1,447	–[a]	1,447	–
1,383	1,384	–[a]	1,383	–
1,360	1,360	1,361	1,361	–
1,335	1,337	1,337	1,336	–
–[a]	1,281	1,282	1,280	1,275
–[a]	1,271	1,271	1,270	–
1,238	1,239	1,239	1,240	1,240
1,191	1,191	1,194	1,197	1,198
1,160	1,160	1,160	1,160	1,165

[a] Spectral region is obscured by solvent absorption

Table 2. Infrared- and resonance Raman-data of 11-*cis* model compounds and rhodopsin (cm^{-1}). Measuring conditions were as in Fig. 5. Resonance Raman-data of 11-*cis*-NRBA-HCl are from Mathies et al. 1977 and of rhodopsin from Mathies et al. 1976

Infrared			Resonance Raman	
11-*cis*-NRBA-HCl in			11-*cis*-NRBA-HCl in EtOH	Rhodopsin
CH_2Cl_2	$CHCl_3$	MeOH		
1,652	1,655	1,659	1,658	1,660
1,605	1,605	1,607	–	1,605
1,556	1,555	1,560	1,556	1,545
1,470	1,470	–[a]	–	–
1,457	1,457	–[a]	–	–
1,448	1,448	–[a]	1,439	1,450
1,430	1,430	–[a]	–	1,430
1,398	1,398	–[a]	–	1,390
1,383	1,384	1,383	–	–
1,375	1,374	1,374	–	–
1,359	1,359	1,359	–	1,358
1,335	1,337	1,335	–	–
–[a]	–	1,275	1,276	1,270
–[a]	1,263	1,260	–	–
1,232	1,233	1,234	1,237	1,240
1,213	–[a]	1,212	1,218	1,216
1,204	1,201	1,208	–	–
1,180	1,180	1,184	1,190	–
1,140	1,141	–	–	–
1,114	1,116	–	–	–

[a] Spectral region is obscured by solvent absorption

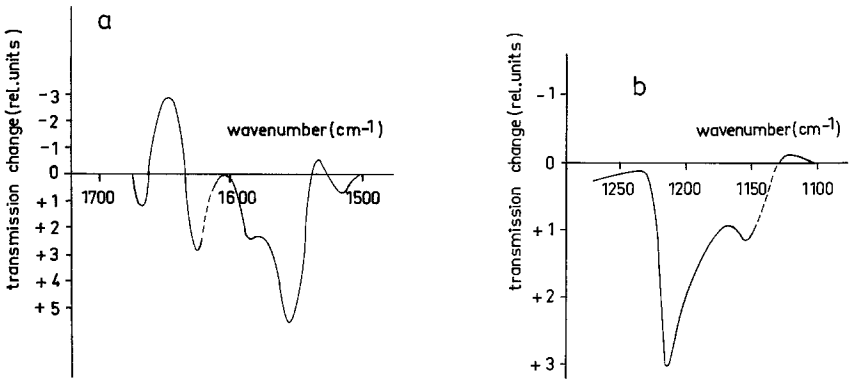

Fig. 6. Meta I/Meta II difference spectrum. Difference spectrum was obtained by subtracting the rhodopsin/Meta I difference spectrum (Fig. 4) from the rhodopsin/Meta II difference spectrum (Fig. 3). Same units are used for relative transmission changes $\Delta I/I$ as in Figs. 3 and 4. **a** 1,680 cm^{-1}–1,500 cm^{-1}; **b** 1,250 cm^{-1}–1,100 cm^{-1}

at 1,191 cm^{-1} of all-*trans*-NRBA-HCl in $CHCl_3$ and at 1,194 cm^{-1} in MeOH, Table 1, whereas the opposite shift would have been expected since λ_{max} is at 460 nm for the former and at 442 nm for the latter).

The vibronic spectrum of 11-*cis*-retinal in rhodopsin is given by the published resonance Raman spectrum of rhodopsin (Mathies et al. 1976; Callender et al. 1976). As has been demonstrated above, however, discrepancies are observed in band positions between infrared spectra and resonance Raman spectra of protonated NRBA. Assuming the same differences prevailing also in rhodopsin, the infrared spectrum of retinal in rhodopsin can be deduced approximately from the resonance Raman spectrum of rhodopsin together with the infrared spectrum of 11-*cis*-NRBA-HCl.

The rhodopsin/Meta II difference spectrum between 1,270 cm^{-1} and 1,140 cm^{-1}, Fig. 3b, resembles the infrared spectrum of 11-*cis*-NRBA-HCl if positive transmission changes are substituted for absorbance. This has been expected from the discussion about the effect of the deprotonation on the infrared spectra of model compounds. Band positions correspond approximately to the resonance Raman data of rhodopsin, if the above mentioned corrections are made. This supports the conclusion from resonance Raman experiments (Mathies et al. 1976; Callender et al. 1976), which show that the influence of the protein on the conformation of the retinal is not very strong and that the Schiff base in Meta II is essentially unprotonated. Only the positive transmission change at 1,150 cm^{-1} appears to be rather large compared with the small peak at 1,140 cm^{-1} of 11-*cis*-NRBA-HCl (Fig. 5).

The large positive transmission changes at 1,390 cm^{-1} and 1,440 cm^{-1} in the rhodopsin/Meta II difference spectrum can, in our opinion, not be explained simply on the basis of the infrared spectrum of protonated 11-*cis*-NRBA or the resonance Raman spectrum of rhodopsin. Indeed, there are bands in this region in the infrared spectrum of 11-*cis*-NRBA-HCl (Fig. 5a and Table 2). They are attributed to the CH_2-deformation vibrations and to the assymmetric and symmetric methyl-deformation vibrations. They are not expected to depend strongly on the isomeric form of the retinals or on the state of protonation of the Schiff base. This is verified by the infrared spectra of the

model compounds (Fig. 5). The small differences between the infrared spectra at those wavenumbers cannot be reconciled with the large changes observed in the difference spectrum. Preliminary measurements of the temperature dependence of the amplitude and rate constant of the rhodopsin/Meta II signal at 1,392 cm^{-1} suggest that it does not reflect the Meta I/Meta II equilibrium. Thus it might represent some molecular changes in the opsin, leading to the light scattering signal of ROS which shows a similar temperature dependence (Hofmann et al. 1976; Wey and Cone 1978).

From the resonance Raman spectrum of rhodopsin and the data of Table 2 the infrared band of the C=C-stretching vibration is expected at 1,550 cm^{-1}. This is in accordance with the large positive transmission change observed in the rhodopsin/Meta II difference spectrum at 1,554 cm^{-1} (Fig. 3a). The shoulder at 1,585 cm^{-1} is probably produced by the appearance of the corresponding band in Meta II, which is weaker and narrower, and shifted to higher wavenumbers in the deprotonated form.

The results of the rhodopsin/Meta II difference spectrum in the region of the C=N-vibration are surprising. From the infrared spectra of 11-*cis*-NRBA-HCl and the data of Table 2 the C=N-vibration should appear as a strong band at 1,660 cm^{-1}, comparable in intensity with the C=C band. This band should be shifted to 1,622 cm^{-1} upon deprotonation and decrease in intensity (Fig. 5a). The resulting difference spectrum should show a large positive transmission change at 1,660 cm^{-1} and a smaller negative transmission change at 1,622 cm^{-1}. Evidently there are discrepancies between the observed and expected difference spectra in this region.

One might argue that the spectrum of the C=N-vibration is disturbed by changes of some other vibration. Especially changes of the amide I-band could interfere, since this band is very strong (Fig. 1a). For the identification of the C=N-vibration it would, therefore, be advantageous to modify selectively the C=N-vibration. The identification of the 1,660 cm^{-1}-band with the C=N-vibration in resonance Raman spectra of rhodopsin and model compounds was mainly made by deuterium exchange experiments in which the proton of the nitrogen was replaced by deuterium, and a corresponding shift to lower wavenumbers, due to the larger reduced mass, was observed (Oseroff and Callender 1974). This method is equally suited in our case, since deuterium exchange does not affect the amide I-band. The difference between the normal and deuterated difference spectra should reveal the protonated and deuterated C=N-vibrations, which should show up as bands of strengths comparable to those of the C=C- and C-C-vibrations (Fig. 5a). The effect of deuterium exchange on the kinetic rhodopsin/Meta II difference spectrum is shown by the dashed line in Fig. 3a. Since the D_2O-absorption does not interfere between 1,700 cm^{-1} and 1,500 cm^{-1} squeezed sediment samples could be used throughout. Evidently the changes induced by deuterium exchange are rather small in comparison with the C=C- and C-C-bands. They can, however, not be explained by imcomplete deuteration, since the decrease of the amide II-band in the survey spectrum showed that the highest degree of deuteration, which can be obtained by deuterium exchange, had been reached (Osborne and Nabedryk-Viala 1977).

Since the rhodopsin/Meta II difference spectrum in the fingerprint region and in the region of the C=C-vibration agrees well with resonance Raman data and with the spectra of model compounds, it is rather unlikely that in those spectral regions the difference spectrum is markedly generated by other than the retinal vibrations. Thus, it appears

justified to use the strength of these bands as a measure for the strength of the C=N-band. We therefore infer that the C=N-vibration shows up essentially different in resonance Raman spectra in comparison with infrared spectra of the retinal in rhodopsin. Preliminary measurements of the bacteriorhodopsin$_{570}$-M$_{412}$-difference spectrum support this view, since they again show good agreement with resonance Raman experiments in the C=C-vibration- and fingerprint-regions, but inconsistencies in the region of the C=N-vibration. A possible explanation for this phenomenon, which requires the results of the rhodopsin/Meta I difference spectrum, will be given after the rhodopsin/Meta I difference spectrum has been discussed.

We wish to emphasize that the deuterated squeezed sediment samples reproduced the results obtained from hydrated films, excepting the effect of deuterium exchange. This again justifies the use of hydrated film samples for measurements near a water absorption band.

Rhodopsin/Meta I Difference Spectrum

The rhodopsin/Meta I difference spectrum between 1,250 cm^{-1} and 1,140 cm^{-1}, Fig. 4b, can be partially explained on the basis of the spectra of the model compounds, Fig. 5a and b, and by the data of all-*trans*-NRBA-$HClO_4$, Table 1, assuming that Meta I represents an all-*trans* protonated Schiff base. A band at 1,237 cm^{-1} in rhodopsin is reduced and, from Fig. 5b, would be shifted to 1,240 cm^{-1}. An additional large band at 1,205 cm^{-1} appears. If one calculates the difference between the rhodopsin/Meta I and rhodopsin/Meta II difference spectra one should obtain the Meta I/Meta II difference spectrum; however, as discussed above, the absorbance of the unprotonated Schiff base in this region is very low. Thus, an approximation of the Meta I spectrum should be obtained. Carrying out this subtraction, a large broad band centered at 1,205 cm^{-1} with a shoulder at 1,155 cm^{-1} is obtained (Fig. 6b). This generated Meta I spectrum resembles the spectrum of all-*trans*-NRBA-$HClO_4$, with the exception of the band at 1,240 cm^{-1} which is missing and the band 1,155 cm^{-1}, which is larger than in the spectrum of the model compound. These discrepancies could perhaps be explained if the retinal in Meta I still exists in some twisted form. It is interesting to note that in resonance Raman spectra of protonated all-*trans*-NRBA the 1,240 cm^{-1}-band is very small (Mathies et al. 1977). Due to a different geometry of retinal in Meta I the infrared activity of this band could be greatly reduced compared to protonated all-*trans*-NRBA. One has, however, to take into consideration that forming the Meta I/Meta II difference spectrum from the measured kinetic difference spectra introduces large errors, which make such a discussion speculative.

The rhodopsin/Meta I difference spectrum in the region between 1,600 cm^{-1} and 1,500 cm^{-1} appears very complex (Fig. 4a). From the negative transmission changes in this spectral region it is evident that Meta I absorbs stronger than rhodopsin. On computing the Meta I/Meta II difference spectrum a single band centered at 1,555 cm^{-1} with a shoulder at 1,580 cm^{-1} is obtained (Fig. 6a). It resembles, with exception of the broadness, the rhodopsin/Meta II difference spectrum. Whether the band in Meta I is shifted as compared to the band in rhodopsin cannot be deduced since the errors introduced by computing the difference spectrum are too large. In any case, no simple

shift of the C=C-vibration due to the change of λ_{max} from 498 nm to 478 nm is observed.

The rhodopsin/Meta I difference spectrum between 1,700 cm^{-1} and 1,600 cm^{-1}, Fig 4a, can be interpreted as if a single band centered in rhodopsin at 1,655 cm^{-1} is shifted in Meta I to 1,645 cm^{-1}. If rhodopsin and Meta I were simply protonated Schiff bases, a smaller shift in the opposite direction would have been expected due to the change in λ_{max} (compare the data in Table 1 for all-*trans*-NRBA-HCl in CH_2Cl_2 and all-*trans*-NRBA-$HClO_4$ in $CHCl_3$, for which $\Delta\lambda_{max}$ is 20 nm). The effect of deuterium exchange on the difference spectrum is very small (dashed line in Fig. 4a). The main difference is at 1,625 cm^{-1}, where in the deuterated sample a small positive transmission change is observed instead of a negative one. Special care was taken when drying the sample to prevent the loss of deuterium exchange, as monitored by the amide II-band. The computed Meta I/Meta II difference spectrum in this spectral region, Fig. 6a, shows that it is very similar to the rhodopsin/Meta II difference spectrum (Fig. 3a). The main differences are: 1. the positive transmission change at 1,665 cm^{-1} and the negative transmission change at 1,645 cm^{-1} are reduced; 2. the positive transmission change at 1,625 cm^{-1} is slightly increased. The effect of deuteration on the Meta I/Meta II difference spectrum appears to be small. Most changes observed in the rhodopsin/Meta II difference spectrum are cancelled by the rhodopsin/Meta I difference spectrum, which means, that rhodopsin is more influenced by deuterium exchange than Meta I. Only the positive transmission change at 1,665 cm^{-1} is reduced. A closer examination of the deuterated rhodopsin/Meta I difference spectrum, however, will require more data. The same arguments which have been given for the discussion of the rhodopsin/Meta II difference spectrum also hold true here: it is not probable that only the small changes observed by deuterium exchange are due to the C=N-vibration.

It appears to us that the rhodopsin/Meta II and rhodopsin/Meta I difference spectra in the region between 1,700 cm^{-1} and 1,600 cm^{-1} cannot be explained simply on the basis of a protonated Schiff base and its deprotonation. We give the following interpretation: The Schiff bases in rhodopsin and Meta I are not protonated in the usual sense but rather by a hydrogen bond. This has recently been suggested by others on the basis of theoretical arguments (Favrot et al. 1978, 1979; Hárosi et al. 1978) and experimental evidence (Peters et al. 1977; Abrahamson et al. 1977). The position of the proton is delocalized due to the hydrogen bond, causing a broad or even split C=N-band. In Meta I the proton is removed slightly from the nitrogen of the Schiff base causing the shift of λ_{max} to 478 nm and the shift of the center of the C=N-band(s) to lower wavenumbers (as complete deprotonation effects a shift of λ_{max} to 360 nm and concomitant a shift of the C=N-band to 1,622 cm^{-1}). This shift of the C=N-band(s) explains the rhodopsin/Meta I difference spectrum and the distinction between the rhodopsin/Meta II and Meta I/Meta II difference spectra. A shift of approximately 10 cm^{-1} can be derived. In rhodopsin, deuterium exchange causes a shift of the center of the C=N-band(s) to lower wavenumbers due to the increased reduced mass. Since in Meta I the proton is further away from the nitrogen, the influence of deuterium exchange on the C=N-vibration would be less than in rhodopsin. In Meta II the Schiff base is supposed to be deprotonated.

It is, at present, not possible to assign unequivocally the deprotonated form of the C=N-vibration to a band in the rhodopsin Meta II- or Meta I/Meta II difference

spectra. For a normal deprotonated Schiff base the negative transmission change at 1,645 cm^{-1} is located at a wavenumber too high to represent the C=N-vibration. Also its intensity appears rather large (compare the IR-spectrum of all-*trans*-NRBA, Fig. 5c). One has, however, to take into account that λ_{max} of all-*trans*-NRBA is at 360 nm, whereas λ_{max} of Meta II is at 380 nm. The reason for this is not clear. It could be caused by some negative charge near the β-ionone ring, which is supposed to be present in rhodopsin (Honig et al. 1976; Favrot et al. 1978). Another possibility would be, that there is still some residual interaction between the Schiff base nitrogen and a proton donating group. The longer wavelength of Meta II could explain the higher wavenumber and higher intensity of the C=N-band. In this connection it is interesting to note that the intensity of the C=N-vibration is less affected by deprotonation than the C=C- and C-C-vibrations (Fig. 5).

It remains to explain why kinetic infrared difference spectroscopy gives results different from those obtained by resonance Raman spectroscopy for the C=N-vibration, whereas for the C=C- and C-C-vibration good agreement is obtained. It might be that, if the Schiff base is protonated via an hydrogen bond as dicussed above, a proton transfer is produced in the excited state involved in resonance Raman scattering. For such systems, however, the theory for resonance Raman scattering is very complicated and one has to take into consideration the time dependent evolution of the excited state after the absorption of a photon. It is conceivable, therefore, that a proton is translocated before the reemission of the photon from the excited state. This would change the Raman spectrum of vibrations in which the proton is involved. Thus, in our opinion, the reported resonance Raman experiments do not necessarily contradict our experimental results and the hypothesis of a Schiff base protonated via an hydrogen bond.

In this connection it is interesting to note that in resonance Raman experiments the frequency shift of the C=N-vibration induced by deuteration does not in all cases coincide with the frequency shift calculated on the basis of the increased reduced mass. Especially for the low frequencies in bacteriorhodopsin$_{570}$ and its photoproduct bO$_{640}$ deviations are observed (Stockburger et al. 1979; Terner et al. 1979). This could be explained by the assumption that a residual hydrogen bond persists in the excited state which would reduce the shifts caused by deuteration.

Conclusions

In many aspects our results confirm the resonance Raman experiments. This holds true especially for vibrations sensitive for the isomeric form of the retinal. On the other hand, this shows that it is possible to apply kinetic infrared spectroscopy to complicated systems where only small parts of a macromolecule, such as retinal in rhodopsin, undergoe molecular changes.

Concerning the problem of the protonation of the Schiff base, raised in the introduction, we have obtained results deviating from resonance Raman experiments. Our results support the view of the Schiff base protonated via an hydrogen bond. The origin of these discrepancies is not clear. It might be, however, that the proposed proton transfer in the excited state could affect the resonance Raman spectrum. On the other hand, this proton transfer could initiate molecular changes in the protein part of

rhodopsin. Additional experiments with higher amplitude and time resolution are required to elucidate the structure of the proposed hydrogen bond.

In addition we have obtained spectral changes which are difficult to assign to the chromophore. The corresponding kinetic signals show a temperature dependence of the amplitude and time course different from the signals reflecting the Meta I/Meta II transition of the chromophore. Thus, we attribute these spectral changes to molecular events in the protein part of rhodopsin. Further work is required to unveil the molecular mechanism leading to these spectral changes.

Acknowledgements. We would like to thank Dr. W. Kreutz for his interest and support of this work, Dr. C. Sandorfy and Dr. M. Stockburger for having made available their papers prior to publication.

References

Abrahamson EW, Wiesenfeld JR (1972) In: Dartnall HJA (ed) Photochemistry of vision. Springer, Berlin Heidelberg New York. (Handbook of sensory physiology, VII/1, pp 69–121)

Abrahamson EW, Shriver J, Fager R, Matescu G, Torchia D (1977) Unprotonated chromophor-protein bond in visual pigments from ^{13}C-NMR-spectra. Nature 270:271–273

Blatz PE, Mohler JH, Navangul HV (1972) Anion induced wavelength regulation of absorption maxima of Schiff bases of retinal. Biochemistry 11:848–855

Callender RH, Doukas A, Crouch R, Nakanishi K (1976) Molecular flow resonance Raman effect from retinal and rhodopsin. Biochemistry 15:1621–1629

Chapman D, Byrne P, Shipley G (1966) The physical properties of phospholipids: I. Solid state and mesomorphic properties of some 2,3-diacyl-DL-phosphatidylethanolamines. Proc R Soc 290:115–142

Chapman D, Williams RM, Ladbrooks BD (1967) Physical studies of phospholipids. VI Thermotropic and lyotropic mesomorphism of some 1,2-diacyl-phosphatidylcholines (Lecithins). Chem Phys Lipids 1:445–475

DeGrip WJ, Bonting SL, Daemen FJM (1975) Biochemical aspects of the visual process XXVIII: Classification of S-H-groups in rhodopsin and other photoreceptor membrane proteins. Biochim Biophys Acta 396:104–115

Falk G, Fatt P (1966) Rapid hydrogen ion uptake of rod outer segments and rhodopsin solutions on illumination. J Physiol (Lond) 183:211–224

Favrot J, Leclercq J-M, Roberge R, Sandorfy C, Vocelle D (1978) The photochemical step of vision. The hydrogen bond and other associations. Chem Phys Lett 53:433–435

Favrot J, Leclercq J-M, Roberge R, Sandorfy C, Vocelle D (1979) Intermolecular interaction in visual pigments. The hydrogen bond in vision. Photochem Photobiol 29:99–108

Fung BKK, Hubbell WL (1978) Proteolysis of rhodopsin in native membranes and the distribution of sulfhydryl groups in the fragments. Biochemistry 17:4396–4402

Hàrosi FI, Favrot J, Leclercq JM, Vocelle D, Sandorfy C (1978) Photochemistry of visual pigments: an interpretation of spectral changes in terms of molecular associations and isomerisation. Rev Can Biol 37:257–271

Heyde ME, Gill D, Kilponen RG, Rimai L (1971) Raman spectra of Schiff bases of retinal (models of visual photoreceptors). J Am Chem Soc 93:6776–6780

Hofmann KP, Uhl R, Hofmann W, Kreutz W (1976) Light induced light-scattering and-absorption changes in outer segments of vertebrate light sensitive rod cells. Biophys Struct Mech 2:61–77

Honig B, Greenberg AD, Dinur U, Ebrey ThG (1976) Visual pigment spectra: Implication of the protonation of the retinal Schiff base. Biochemistry 15:4593–4599

Lewis A, Fager RS, Abrahamson EW (1973) Tunable laser resonance Raman spectroscopy of the visual process. J Raman Sepctrosc 1:465–470

Mathies R, Oseroff AR, Stryer L (1976) Rapid-flow resonance Raman spectroscopy of photolabile molecules: rhodopsin and isorhodopsin. Proc Natl Acad Sci USA 73:1–5

Mathies R, Freedman TB, Stryer L (1977) Resonance Raman studies of the conformation of retinal in rhodopsin and isorhodopsin. J Mol Biol 109:367–372

Osborne HB, Nabedryk-Viala E (1977) The hydrophobic heart of rhodopsin revealed by an infrared ^{1}H-^{2}H-exchange study. FEBS Lett 84:217–220

Oseroff AR, Callender RH (1974) Resonance Raman spectroscopy of rhodopsin in retinal disc membranes. Biochemistry 13:4243–4248

Peters K, Applebury ML, Rentzepis PM (1977) Primary photochemical event in vision: proton translocation. Proc Natl Acad Sci USA 74:3119–3123

Rafferty CN (1979) Light-induced pertubation of aromatic residues in bovine rhodopsin and bacteriorhodopsin. Photochem Photobiol 29:109–120

Rafferty CN, Cassim JY, McConnell DG (1977) Circular dichroism, optical rotatory dispersion and absorption studies on the conformation of bovine rhodopsin in situ and solubilized with detergent. Biophys Struct Mech 2:277–320

Rimai L, Heyde ME, Gill D (1973) Vibrational spectra of some carotenoids and related linear polyenes. A Raman spectroscopic study. J Am Chem Soc 95:4493–4501

Robeson CD, Blum WP, Dieterle JM, Cawley JD, Baxter JG (1955) Chemistry of vitamin A: XXV Geometrical isomers of vitamin A aldehyde and an isomer of its α-ionone analogue. J Am Chem Soc 77:4120–4125

Rowan III R, Warshel A, Sykes BD, Karplus M (1974) Conformation of retinal isomers. Biochemistry 13:970–981

Siebert F, Schmid H, Mull RH (1977) Proteins of bovine rod outer segments characterized by SDS-polyacrylamide gel electrophoresis. Biochem Biophys Res Commun 75:1071–1077

Siebert F, Mäntele W, Kreutz W (1980) Flash induced kinetic infrared spectroscopy applied to biochemical systems. Biophys Struct Mech 6:139–146

Sperling W (1973) In: Langer H (ed) Biochemistry and physiology of visual pigments. Springer, Berlin Heidelberg New York, pp 19–28

Stockburger M, Klusmann W, Gattermann H, Massig G, Peters R (1979) The photochemical cycle of bacteriorhodopsin studied by resonance Raman spectrosopy. Biochemistry 18:4886–4900

Terner J, Chung-Lu Hsieh, Burns AR, El-Sayed MA (1979) Time resolved resonance Raman characterization of the bO_{640} intermediate of bacteriorhodopsin. Reprotonation of the Schiff base. Biochemistry 18:3629–3634

Uhl R, Hofmann KP, Kreutz W (1978) On the light stimulated coupling between rhodopsin and its disc membrane environment. Biochemistry 17:5347–5352

Wald G, Durrel J, George RCCSt. (1950) The light reaction in the bleaching of rhodopsin. Science 111:179–181

Wey CL, Cone RA (1978) Light induced light scattering changes from rod outer segments. Biophys J 21:Abstr. TU-POS-D6

Yoshizawa T (1972) In: Darthall HJA (ed) Photochemistry of vision. Springer, Berlin Heidelberg New York. (Handbook of sensory physiology, VII/1, pp 146–179)

Received June 21, 1979/Accepted February 1, 1980