Subpicosecond resonance Raman spectra of the early intermediates in the photocycle of bacteriorhodopsin

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ABSTRACT The resonance Raman spectra are presented for the species formed during the photocycle of bacteriorhodopsin (bR) on a timescale of 800–900 fs. In the ethylenic stretch region two intermediates were found with frequencies of 1,510 and 1,518 cm^{-1}, corresponding to species with optical absorption maxima at 660 and 625 nm, respectively. This leads to the assignment of the 1,518 cm^{-1} band to the J_{625} intermediate. In the fingerprint region, the appearance of a vibration at 1,195 cm^{-1} strongly suggests that the isomerization indeed has taken place in a time less than the pulsewidth of our laser. This supports the previous proposals made on the basis of the optical spectra. The spectra are compared with those observed in tens of picoseconds up to nanoseconds.

INTRODUCTION

Upon absorption of light, the membrane protein bacteriorhodopsin (bR) pumps protons across the cell membrane of *Halobacterium halobium,* thus producing an electrochemical gradient that drives the bacteria's metabolic processes (Stoeckenius and Bogomolni, 1982). Excitation of the bR chromophore, retinal, initiates a photocycle which is completed within 10 ms at room temperature. The kinetics of this cycle, which consists of intermediates with lifetimes varying from femtoseconds to milliseconds, have been extensively studied with optical transient absorption techniques (Lozier et al., 1975; Kaufmann et al., 1976). The different intermediates are labeled by a letter and a number, which is their approximate absorption maximum:

$$bR_{570} \rightarrow J_{625} \rightarrow K_{610} \rightarrow L_{550} \rightarrow M_{412} \rightarrow O_{640} \rightarrow bR_{570}.$$

The retinal is connected to a lysine residue of the protein backbone through a protonated Schiff base (PSB) linkage. This PSB deprotonates during the $L_{550} \rightarrow M_{412}$ step, leading to the proton pumping process, and reprotonates in the following step.

The primary photochemical event in the photocycle has been a subject of considerable interest. Early picosecond optical studies suggested the formation of a red-shifted primary photoproduct K_{610} in 11 ps (Applebury et al., 1978). In later studies a number of experiments showed a redshifted precursor of K_{610} which forms in ~500 fs and relaxes to K_{610} on a 3 ps timescale (Sharkov et al., 1985; Nuss et al., 1985; Polland et al., 1986; Mathies et al., 1988; Dobler et al., 1988). This precursor was labeled J_{625}, although there was some uncertainty about its absorption maximum.

Although transient absorption spectra are obtainable in the short-time domain, they are generally very broad and structureless, and give little or no information on configurational or conformational changes. Time-resolved resonance Raman spectroscopy of picosecond transients was demonstrated in 1980 by Terner et al. for the carboxyhemoglobin photointermediate and in 1981 by Hsieh et al. for bR. The vibrational spectra are greatly simplified when the laser is tuned to the electronic absorption wavelength of the retinal, because only retinal vibrations which mimic a molecular distortion in the excited state are enhanced. Using microbeam and flow techniques with picosecond excitation and optical multichannel analyzer (OMA) detection, it has proven possible to detect the resonance Raman spectra of picosecond transients (for a review see Terner and El-Sayed, 1985).

Bacteriorhodopsin has been the subject of a great number of resonance Raman studies. It has been shown that upon light absorption an *all-trans* to *13-cis* isomerization occurs in the retinal. This isomerization has already taken place in K_{610}, i.e., before 10–40 ps (Hsieh et al., 1981; Stern and Mathies, 1985; Atkinson et al., 1989). The resonance Raman spectrum of K_{610} was further thoroughly studied up to 25 ns (Smith et al., 1983; Hsieh et al., 1983; Stern and Mathies, 1985), but essentially similar results were obtained. Whether isomerization has already taken place in the J_{625} intermediate has been an open question. However, from a time-resolved pump/probe study of bR reconstituted with a sterically hindered retinal, 9,12-phenyl-retinal, using 3-ps pulses Polland et

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al. (1984) concluded that formation of the K_{610} intermediate in bR is accompanied by an *all-trans* to *13-cis* isomerization. The pulsewidth used might allow for a similar conclusion on J_{625}.

In this paper we present the first resonance Raman spectrum which can exclusively be attributed to J_{625}. Spectra of the different vibrational regions were recorded. It is shown by a study of the "fingerprint region", which contains the vibrations sensitive to the isomeric configuration of the retinal, that isomerization occurs in a time interval equal to or shorter than the photolyzing laser pulse of 800–900 fs. In the ethylenic stretch region, apart from a peak at 1,518 cm^{-1} corresponding to the J_{625} intermediate, another species is found at this timescale showing a shoulder at ~1,510 cm^{-1} corresponding to a λ_{max} of 660 nm. The results are discussed and compared with spectra reported in the literature on a timescale varying from femto- to nanoseconds.

MATERIALS AND METHODS

Sample preparation

The purple membrane was purified from the ET1-001 strain of *Halobacterium halobium* according to the method of Oesterhelt and Stoeckenius (1974) and Becher and Cassim (1975). The purified purple membrane was suspended in doubly deionized water at a concentration of ~60 μM as measured by the retinal absorption at 570 nm.

Experimental set-up

The experimental arrangement for obtaining the subpicosecond resonance Raman spectra is similar to the one first reported by Terner et al. (1979*a* and *b*) and Hsieh et al. (1983), the only difference being that another laser system was used with a shorter pulsewidth.

In the present work a cw Nd:YAG laser (model 3800, Spectra-Physics Inc., Mountain View, CA), mode-locked at 82 MHz, produces a train of ~80-ps pulses at an average power of 14 W. The beam was directed into a Spectra Physics fiber-grating pulse compressor (model SP 3695), which shortens the 1,064-nm pulses to <6 ps. An SP 3225 frequency doubler is directly mounted in the compressor, producing ~1.2 W at 532 nm. The green output, which is further stabilized to ~800 mW by an acousto-optic stabilizer (model SP 3275), is used to synchronously pump a Spectra Physics 375B dye-laser, with Rhodamine 6G in ethylene glycol, fitted with an SP 344S cavity dumper. A two-plate birefringent is used as a wavelength tuning device. The bandwidth of the dye-laser is ~16–18 cm^{-1}. At a dumping rate of 800 kHz, the average output power of the dye-laser system at the excitation wavelength of 587 nm was 25 mW (31 nJ/pulse). This wavelength was selected to simultaneously increase the photolysis quantum yield and to make efficient use of the resonance Raman enhancement. Furthermore it should be noted that the red fluorescence of bR obscures the resonance Raman signals when much longer wavelengths are used. The power used is sufficiently small to minimize stimulated processes. The temporal width of the excitation pulse was ~800–900 fs, as measured by autocorrelation (model 5-14A, Inrad Inc., Northvale, NJ) assuming a $sech^2$ pulseshape.

The beam was directed via a microscope objective (Zeiss Neofluar 40×) onto a vertically flowing free jet of bR suspension, and was thus tightly focused to a ~10-μm spot. The sample was kept light-adapted by a lamp and was circulated from the sample vial through a syringe needle with a 110-μm diameter by a peristaltic pump (Masterflex, Cole-Parmer Instrument Co., Chicago, IL). The measured flow rate was 25 m/s which, combined with the 10-μm spotsize, gave a sample residence time of 0.4 μs. Because the time between the laser pulses is 1.25 μs, each pulse should have encountered a fresh volume of unphotolyzed light-adapted bR.

The scattered light was collected at 90° and focused onto the slit of a model 1870 0.5-m spectrograph (Spex Industries Inc., Edison, NJ), and the Rayleigh scattering was blocked by appropriate Corning cut-off filters (2-61 and 2-62 for the ethylenic stretching region; 2-62 and 2-63 for the fingerprint region). The resolution of the monochromator was determined to be 3 cm^{-1}. The detection system consisted of a diode array detector, thermoelectrically cooled to −20°C (model 1455, EG&G Princeton Applied Research Corp., Princeton, NJ) and an OMA (model 1460) with a 1462 detector controller and 1462/99 14-bit A/D controller (EG&G Princeton Applied Research Corp.).

Wavelengths were calibrated with the lines of a neon discharge lamp. Vibrational frequencies are estimated to be accurate to ±3 cm^{-1} in the original spectra and to ±5 cm^{-1} in the computer-generated "difference" spectra (see below).

Measurement procedure

In a single laser resonance Raman experiment on bR, each laser pulse serves both as the photolysis and Raman excitation light source. A spectrum of the unphotolyzed bR_{570} sample is obtained at a low average laser power. The excitation conditions can be described in terms of a photolability parameter $F = I_0 \cdot t$ (Mathies et al., 1976; Atkinson et al., 1985), in which t is the residence time of the sample in the laser beam and

$$I_0 = \phi \cdot \sigma_{bR}(\lambda) \cdot I(\lambda, r), \quad (1)$$

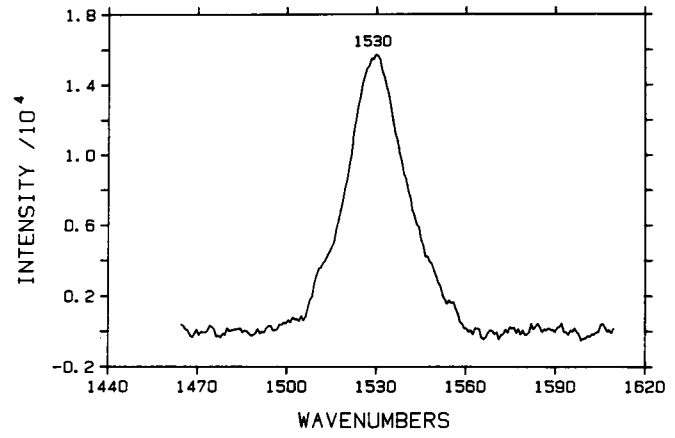
in which $\sigma_{bR}(\lambda)$ is the absorption cross-section of bR_{570} at the excitation wavelength, ϕ is the photochemical quantum yield for reaction, and $I(\lambda, r)$ is the laser intensity. The latter can be calculated from the peak power of the laser and the diameter of the focused laser spot in the jet. F was varied between 0.15 and 1.9 by attenuating the average laser power with neutral density filters. At low F values ($F < 0.2$) a spectrum similar to that of bR_{570} is obtained, whereas higher F values ($F > 1$) are needed to produce significant amounts of photoproducts.

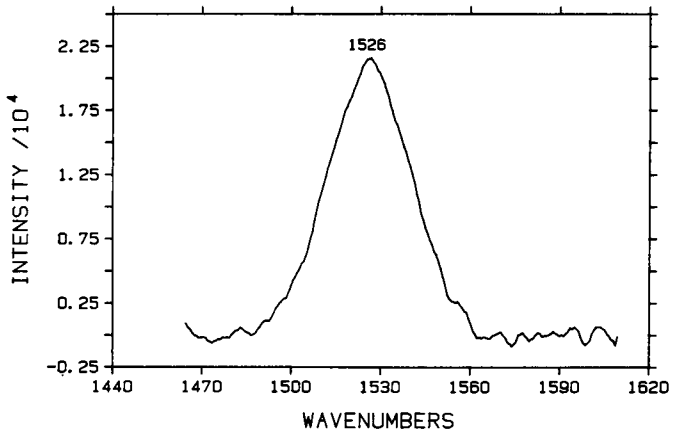
The "difference" spectra were obtained by subtracting an arbitrarily scaled low-power spectrum from a high-power spectrum. As was noted by Hsieh et al. (1983) caution should be exercised when information is extracted from these "difference" spectra. They are to be used strictly as a guide to interpreting the actual spectral changes.

RESULTS AND DISCUSSION

Resonance Raman spectrum of bR_{570}

To identify the resonance Raman spectra of the photocycle intermediates of bR, an unambiguous, well-characterized spectrum of bR_{570} should be obtained first. Very low laser powers (corresponding to F values of ~0.1–0.2) were used to record such a spectrum, which is shown in Figs. 1 and 2 (upper spectra) for two different spectral regions: 1,450–1,600 cm^{-1} and 900–1,400 cm^{-1}, respectively. The





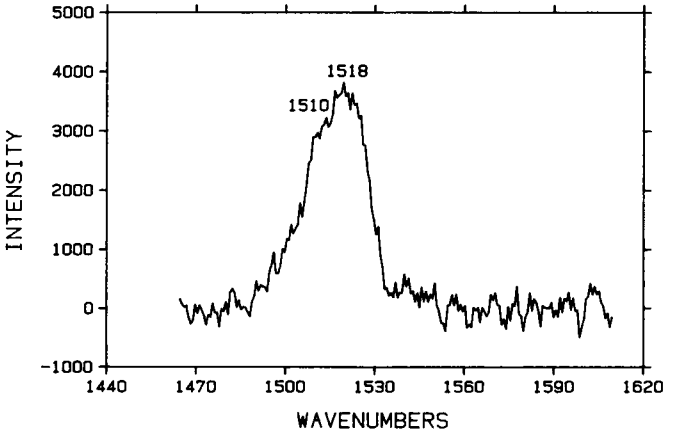
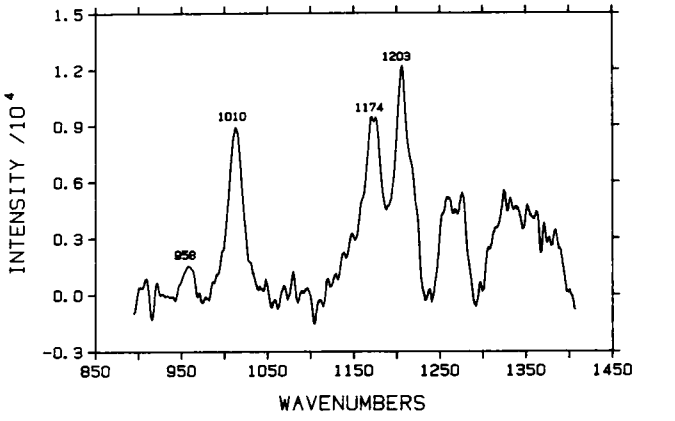
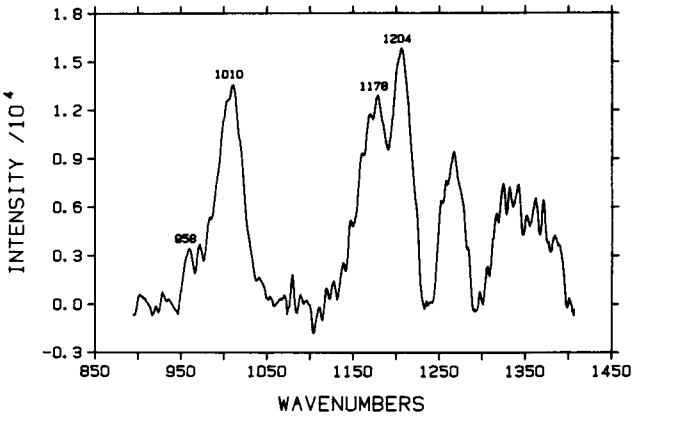


FIGURE 1 Resonance Raman spectra of bR in the ethylenic stretch region around 1,530 cm^{-1}. The upper spectrum was obtained at low laser power ($F = 0.15$) and is the result of unphotolyzed, ground-state bR_{570} only. The middle spectrum was recorded at high laser power ($F = 1.9$) and represents the contributions of both bR_{570} and J_{625}. A clear broadening and shift are observed, showing J_{625} has a lower ethylenic stretching frequency than bR_{570}. The lower part shows the difference spectrum of the high and low laser power spectra. The $\nu_{C=C}$ of the J_{625} intermediate is observed around 1,518 cm^{-1}, in accordance with a linear relation between the absorption maximum of the intermediate and this ethylenic stretching frequency. The occurrence of a shoulder ~1,510 cm^{-1} may indicate the formation of a more red-shifted intermediate with an absorption maximum at 660 nm.





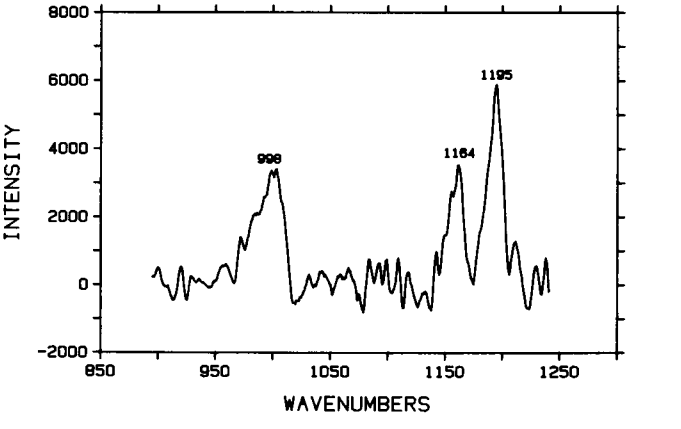


FIGURE 2 Resonance Raman spectra of the fingerprint region of bR. The upper spectrum represents the contribution of unphotolyzed, ground-state bR_{570} only, because it was obtained at very low laser powers. The middle spectrum clearly shows the effects of the initiation of the photocycle at high laser powers and represents a spectrum of bR_{570} and J_{625}. The retinal *all-trans* to *13-cis* isomerization has taken place as can be seen from the filling in of the valley between the 1,174 and 1,203 cm^{-1} peaks. Furthermore, an increase in the region between 960 and 1,000 cm^{-1} can be distinguished. The lower part shows the difference spectrum of the high and low laser power spectra of Fig. 2. The isomerization of the retinal is evident from the peak at 1,195 cm^{-1}. The 1,164 cm^{-1} vibration, which is unique to the J_{625} spectrum, shows that the isomeric configuration at these early times (800–900 fs) is clearly different from that of the K_{610} intermediate.

latter contains the C—C stretches and C—C—H in-plane bends between 1,100 and 1,300 cm^{-1}, which characterize the isomeric configuration of the retinal (Smith et al., 1987*a*), and the hydrogen-out-of-plane (HOOP) and $C-CH_3$ rocking vibrations, between 900 and 1,100 cm^{-1}, which are sensitive to conformational distortions along the C_7-C_{15} hydrocarbon chain (Smith et al., 1987*a*). The region around 1,500 cm^{-1} has been assigned to a C=C stretching mode. Its frequency has been shown to correlate linearly with the electronic absorption maximum of the chromophore (Rimai et al., 1973; Aton et al., 1977).

The bR_{570} spectrum, shown in Figs. 1 and 2 (upper panels) is in agreement with spectra reported previously (Terner et al., 1979*a* and *b*; Stern and Mathies, 1985; Atkinson et al., 1985, 1989). Moreover, a complete vibrational assignment has been given by Smith et al. (1987*a*), based on an extensive set of ^{13}C- and ^{2}H-labeled derivatives. Many of the vibrational features in the bR_{570} spectrum are characteristic of an *all-trans* structure, although each frequency is found to be ~10 cm^{-1} higher than the corresponding frequency in the *all-trans* retinal protonated Schiff base. This was explained as a result of increased π-electron delocalization of the retinal in the protein.

Resonance Raman spectrum in the ethylenic stretch region

The spectrum obtained in the region around 1,500 cm^{-1} at a high value of *F* is shown in Fig. 1 (*middle*). Photolysis is evident from the clear broadening of the 1,526 cm^{-1} band compared to the 1,530 cm^{-1} band in the spectrum of unphotolyzed bR_{570}. Fig. 1 (*bottom*) shows the intense residual band around 1,518 cm^{-1} obtained by subtracting the upper spectrum of Fig. 1 from the middle.

It is known that a linear relation exists between the absorption maximum of a bR photocycle intermediate and the frequency of its ethylenic stretch mode (Rimai et al., 1973; Aton et al., 1977). In Fig. 3 this relation is shown for the different bR intermediates. It should be noted, that some uncertainty exists regarding the $\nu_{C=C}$ of both O_{640} (Terner et al., 1979*b*; Smith et al., 1983) and K_{610} (Terner et al., 1979*a*; Smith et al., 1983; Stern and Mathies, 1985). We have decided to take average values, and plot error bars according to the minimum and maximum values. From this figure the C=C stretch frequency of 1,518 cm^{-1} as found from the difference spectrum of Fig. 1 (*bottom*) would correspond to an intermediate with an absorption maximum of 625 nm and might therefore be attributed to J_{625}. Furthermore, it should be noted that the 1,518 cm^{-1} band is asymmetric on the low-frequency side with a shoulder ~1,510 cm^{-1}. This frequency corresponds to an intermediate with an absorption maximum at 660 nm and might indicate that

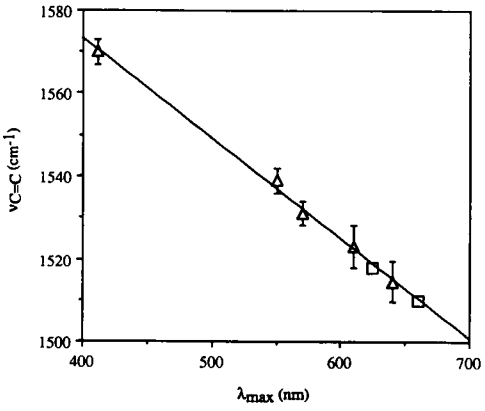


FIGURE 3 Plot of the dependence between the frequency of the ethylenic stretch mode ($\nu_{C=C}$) and the absorption maximum (λ_{max}) of the different bR photocycle intermediates. The intermediates that have been plotted are: M_{412} (1,570 cm^{-1}), L_{550} (1,539 cm^{-1}), bR_{570} (1,531 cm^{-1}), K_{610} (1,523 cm^{-1}) and O_{640} (1,514 cm^{-1}). The C=C stretch frequencies of 1,510 and 1,518 cm^{-1} would correspond to absorption maxima of 660 and 625 nm, respectively. They have been put in the figure with different symbols. A linear relation is obtained.

during the 800–900-fs photolysis pulse either another intermediate is formed, which has an even more red-shifted absorption spectrum than J_{625}, or that an intermediate is formed corresponding to J_{625} from another type of bR.

A large number of studies have been devoted to the earliest intermediates in the bR photocycle. Applebury et al. (1978) observed a red-shifted (625 nm) precursor of K_{610} within the 6-ps time width of the excitation pulse, and Polland et al. (1986) observed an absorption maximum of J_{625} around 615 nm. Further investigations clearly established the formation (~0.5 ps) and decay times (~3 ps) of J_{625} and agreed that its absorption is red-shifted compared to that of K_{610}. However, no spectra were shown (Nuss et al., 1985; Sharkov et al., 1985; Dobler et al., 1988). Very recently, Mathies et al. (1988) carried out a careful time-resolved transient absorption/emission study using 60-fs pump and 6-fs probe pulses, which shows an increased absorption around 640–660 nm on a timescale of 200–900 fs.

The absorption maximum which has been attributed to J_{625} seems to be shifted to the red when it is probed at shorter timescales. At times varying between 1 and 3 ps the absorption maximum seems to be around 625 nm, the value commonly assumed for the J intermediate. The C=C stretch frequency of 1,518 cm^{-1} would then correspond to J_{625}, which would be in complete accord with the linear relation between λ_{max} and $\nu_{C=C}$ (Fig. 3) (Rimai et al., 1973; Aton et al., 1977). At earlier times, however, as shown by the differential transmittance spectra of Mathies et al. (1988) there seems to be a redshift: at short times

(~220 fs) there is an absorption around ~660 nm, whereas at longer times (~990 fs), the transient absorption has shifted to ~630–640 nm. It is therefore possible that the 1,510 cm^{-1} band is due to an absorption of an intermediate before J_{625}, which absorbs around 660 nm and is present on the 200–800 fs timescale. It could also be due to the corresponding J_{625} intermediate in a parallel cycle, that has an absorption in K_{610} not very different from that of the first cycle. The Raman spectrum of L_{550} indeed shows two types of L which are now believed to result from parallel cycles (Diller and Stockburger, 1988). The presence of two types of bR was first suggested from the observation of two risetimes of the M_{412} intermediate (Hanamoto et al., 1984).

Resonance Raman spectrum in the fingerprint region

A "high-power" spectrum in the fingerprint region is shown in the middle part of Fig. 2. A substantial amount of photochemistry has been induced at this higher power, producing a significant concentration of J_{625}. This can be seen from the increased scattering in the region between 960 and 1,000 cm^{-1}. These frequencies are in the HOOP region and are particularly sensitive to conformational distortions along the polyene chain (Smith et al., 1987*a*). A similar observation was made by Atkinson (Atkinson et al., 1985; Brack and Atkinson, 1989) on a timescale of ~10 ps and was interpreted as arising from a twisting motion of the conjugated polyene chain. The observed scattering enhancement of these C—H vibrations would occur when the σ and π^* electron systems become intermixed upon twisting of the polyene chain (Hsieh et al., 1981). The broadening to the low-frequency side might suggest that scattering occurs from hot unrelaxed vibrational levels, as was observed in the C=C stretching region (Brack and Atkinson, 1990). Furthermore, as was observed before (Hsieh et al., 1981), slight differences in the unrelaxed protein environment around the twisted retinal might also induce further broadening in this region.

A variety of experiments have argued that the initial photochemistry in bR involves an *all-trans* to *13-cis* isomerization of the retinal. Such an isomerization has already taken place in the J_{625} intermediate as shown by the filling in of the valley between the 1,174 and the 1,203 cm^{-1} peaks, i.e., the appearance of a new band around 1,195 cm^{-1}. It is this vibrational region which is sensitive to the isomeric state of the retinal chromophore. In the difference spectrum of Fig. 2 (*bottom*) this new band is clearly resolved and so is another band at a lower frequency of 1,164 cm^{-1}. The latter confirms the observaappearing in their 8-ps resonance Raman spectrum. Stern and Mathies (1985) observed this same band on a 7-ps timescale at 1,163 cm^{-1}, but attributed it to differences in the excitation wavelength. This seems to be unlikely, because it has already been shown that the bR_{570} spectrum undergoes only minor changes in relative intensities, when excited at four different wavelengths: 676 nm (Braiman and Mathies, 1982), 568 nm (Terner et al., 1979*a*), 514 nm (Stockburger et al., 1979) and 457 nm (Marcus and Lewis, 1978). A similar observation was made in the resonance Raman spectra of the retinal protonated Schiff base when excited at 413 nm (Braiman and Mathies, 1980) and at 647 nm (Aton et al., 1977). Furthermore, Atkinson et al. (1989) clearly showed that when exciting around 590 nm, the 1,163–1,164-cm^{-1} band is not part of the K_{610} spectrum. However, both the 8-ps spectrum of Atkinson (Atkinson et al., 1989) and the 7-ps spectrum of Stern and Mathies (1985) contain a considerable contribution from K_{610}, because the decay time of J_{625} is 3 ps. This contribution could not be resolved. The spectrum presented here is the first that can be exclusively attributed to J_{625}, because the 800–900-fs pulsewidth does not allow for appreciable K_{610} formation. Furthermore it has been shown that resonance Raman scattering from the electronic excited state of bR_{570} is unlikely, because of its low concentration (due to the extremely short lifetime of ~200 fs) and its small absorption coefficient at the laser wavelength (Polland et al., 1986).

The occurrence of the 1,195 cm^{-1} band is indicative of isomerization at the C_{13}=C_{14} position, i.e., from *all-trans* to *13-cis* retinal. This band was also observed in spectra obtained on a 40-ps (Hsieh et al., 1981, 1983), a 200-ps (Stern and Mathies, 1985), and a 20–30-ns timescale (Hsieh et al., 1983; Smith et al., 1983; Stern and Mathies, 1985). It is also observed for the K_{610} intermediate which Braiman and Mathies (1982) have shown to have a *13-cis* configuration. The 1,164-cm^{-1} vibration on the other hand was not observed in any of the spectra mentioned above, and therefore does show that the retinal isomerization at these very early times (<1 ps) is of a different nature than at much longer times. It is, however, very difficult to determine the exact configuration at C_{13}=C_{14}, because there are many factors which influence the features appearing in the fingerprint region. bR_{570}, which contains an *all-trans* retinal, has a band at ~1,171 cm^{-1}. If one assumes that upon isomerization its frequency changes with time, one observes the following. In our spectrum we observe a transient at 1,164 ± 3 cm^{-1} (λ_{exc} = 587 nm). This same band might be found at 1,163 cm^{-1} in 7 ps (λ_{exc} = 576 nm) (Stern and Mathies, 1985), at 1,170 cm^{-1} at times >10 ps (λ_{exc} = 590 nm) (Brack and Atkinson, 1989), at 1,178 cm^{-1} in 40 ps (λ_{exc} = 587 nm) (Hsieh et al., 1981, 1983), and at 1,185 cm^{-1} in 0.2 ns (λ_{exc} = 514 nm) (Stern and Mathies, 1985) or longer: 20–30 ns (λ_{exc} = 587 nm) (Hsieh et al., 1983), (λ_{exc} =

514 nm) (Stern and Mathies, 1985) and 100 ns (λ_{exc} = 552.3 nm) (Hsieh et al., 1981). Realizing that *13-cis* retinal in dark-adapted bR has a strong band at 1,183 cm^{-1} (Terner et al., 1979*c*; Smith et al., 1987*b*) one is tempted to suggest that the 1,164-cm^{-1} vibration in *all-trans* retinal in bR changes into the 1,185 cm^{-1} vibration during the isomerization process. If so, then the isomerization starts in <500 fs (Dobler et al., 1988; Mathies et al., 1988) and is completed in <200 ps. In that case, the fact that no corresponding changes in the optical absorption of the retinal have been reported on this timescale might suggest that this bond is very sensitive to the protein environment, and that the time dependence of its frequency simply reflects the fast relaxation rates of the amino acid residues within the active site. Its frequency in these early intermediates should therefore be sensitive to genetic substitution of the residues known to be present within the active site (Mogi et al., 1989).

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