Influence of the 9-Methyl Group of the Retinal on the Photocycle of Bacteriorhodopsin Studied by Time-Resolved Rapid-Scan and Static Low-Temperature Fourier Transform Infrared Difference Spectroscopy†

Olaf Weidlich,‡ Noga Friedman,§ Mordechai Sheves,§ and Friedrich Siebert*,‡

Institut für Biophysik und Strahlenbiologie, Albert-Ludwigs-Universität, Albertstrasse 23, D-79104 Freiburg, Germany, and Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel

Received January 11, 1995; Revised Manuscript Received July 18, 1995⊗

ABSTRACT: The photocycle of bacteriorhodopsin (BR) regenerated with *all-trans*-9-demethylretinal was investigated by time-resolved rapid-scan Fourier transform infrared difference spectroscopy, by static low-temperature difference spectroscopy at 80, 170, and 213 K and by static steady-state difference spectroscopy at 278 K. In addition, the formation and decay of M intermediate was monitored at 412 nm with conventional flash photolysis experiments. Our data show that the removal of the 9-methyl group strongly changes the photocycle of BR. The reaction cycle is slowed down about 250-fold. The photoreaction is characterized by a slow rise of the M intermediate and by a very long-lived N intermediate. No O intermediate could be observed. The low-temperature spectra indicate that already at 80 K a KL-like photoproduct is formed. L can be obtained as in native BR at 170 K, but its decay appears to be inhibited, since it can still be observed at 213 K and high pH, in addition to the M intermediate. As in native BR, the 15-hydrogen out-of-plane modes of the L and N intermediates (observed in ^2H_2O) are very similar. Evidence for the existence of three N substates which differ in the protonation state of Asp96 and in the amide I bands is presented. This is explained by the extremely slowed-down reisomerization of the chromophore. The results are discussed with respect to alterations in the chromophore−protein interaction, caused by the removal of the 9-methyl group.

The retinal protein bacteriorhodopsin (BR)[1] pumps protons upon light absorption across the purple membrane of *Halobacterium halobium* [for recent reviews see Khorana (1988), Oesterhelt et al., (1992), Rothschild (1992), Lanyi (1992, 1993), and Ebrey (1993)]. It consists of the protein opsin and the light-sensitive chromophore *all-trans*-retinal that is bound to the ϵ-amino group of Lys216 via a protonated Schiff base. BR is composed of seven transmembrane helices, and a model for its structure has been developed based on images obtained by electron cryomicroscopy (Henderson et al., 1990). Photon absorption by light-adapted BR (BR^{568}) initiates a photocycle which relaxes within a few milliseconds to the initial ground state. The intermediates of the photocycle J, K, KL, L, M, N, and O, appearing in the given order, are characterized by their absorption spectra and lifetimes and by the isomerization and protonation state of the chromophore and the protonation states of internal aspartic acids. The primary step of the photocycle is the isomerization of the chromophore to the 13-*cis* geometry (Tsuda et al., 1980; Braiman & Mathies, 1980, 1982). An essential intermediate is the M state, in which the Schiff base becomes deprotonated (Aton et al., 1977; Marcus & Lewis, 1977) and the proton is transferred to Asp85 (Braiman et al., 1988a; Fahmy et al., 1992). Most models assume that it is the Schiff base proton which is being pumped. In the following N intermediate, the Schiff base picks up the proton from Asp96 (Gerwert et al., 1989; Bousché et al., 1991; Maeda et al., 1992). Thermal reisomerization back to the *all-trans* geometry occurs during the N → O transition (Smith et al., 1983). Asp96 is found to be reprotonated in the O intermediate at pH 6.5 (Hessling et al., 1993; Bousché et al., 1992). The reprotonation of Asp96 (or of another group on the cytoplasmic side) during the lifetime of the N intermediate at high pH has recently been deduced from time-resolved UV−vis measurements combined with experiments using a pH indicator dye (Zimányi et al., 1993; Cao et al., 1993a). De- and reprotonation of the Schiff base is caused by pK_a changes of the Schiff base and/or of Asp85 and Asp96. Thus, specific pK_a changes during the BR to L and M to N transitions are prerequisites for Schiff base deprotonation and reprotonation.

Besides pK_a changes, changes in the accessibility of the Schiff base are required, which prevent proton uptake from the same side to which it had been ejected. Such a "molecular switch" has been postulated (Ames & Mathies, 1990), which involves a quasi-irreversible step ($k_{forward} \gg k_{backward}$) in the photoreaction. Evidence for such a step has been provided by a kinetic analysis of the L decay and M rise, linking two substates M_1 and M_2 of the M intermediate (Váró & Lanyi, 1990). Experiments showing more directly the existence of two M states have recently been published (Váró & Lanyi, 1991; Váró et al., 1992; Zimányi et al., 1992a,b; Druckmann et al., 1992). The difference between the two M's is mainly caused by different protein states. The relaxation to the initial state, i.e., the closing of the cycle,

† Work supported by Deutsche Forschungsgemeinschaft, Az Si-278/6-3

* To whom correspondence should be addressed.
‡ Albert-Ludwigs-Universität.
§ Weizmann Institute of Science.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1995.

[1] Abbreviations: FT-IR, Fourier-transform infrared; UV−vis, ultraviolet−visible; BR, bacteriorhodopsin; 9-H-BR, 9-demethylbacteriorhodopsin; HOOP, hydrogen out-of-plane.

0006-2960/95/0434-13502$09.00/0

involves the isomerization of the chromophore from 13-*cis* to *all-trans*. This step must be driven by the retinal−protein interaction. Evidence for structural changes of the protein during transitions to the various intermediates has been obtained from the large number of published FT-IR experiments [see, e.g., Siebert (1993) for review]. In addition, structural changes upon formation of the M intermediate have been deduced from several diffraction studies (Dencher et al., 1989; Nakasako et al., 1991; Subramaniam et al., 1993). All these structural changes are ultimately caused by the isomerization of the chromophore and the subsequent interactions of the twisted 13-*cis*-retinal with the protein. It is an intriguing idea that the chromophore−protein interaction, which causes the molecular switch and the backisomerization, is influenced by the methyl groups of the retinal. The consequences of the removal of the 9-methyl group on the photoreaction of the visual pigment rhodopsin have been demonstrated (Ganter et al., 1989): Deprotonation of the Schiff base is blocked, and the activation of transducin is greatly diminished. However, only a few studies on the dynamics of artificial BR pigments have been reported; mainly of 13-demethyl-BR (Schiffmiller et al., 1985; Fendler et al., 1987; Zinth et al., 1988; Noguchi et al., 1990; Brack et al., 1992), of active-site-methylated BR (Longstaff & Rando, 1987; Govindjee et al., 1988; Kräutle et al., 1990), and of BR6.11 (Brack et al., 1993).

Since removal of the 9-methyl group has a drastic effect on rhodopsin function, the influence of this group on the function of BR is investigated in this paper by studying the artificial pigment 9-demethyl BR (9-H-BR). It has been reported that the proton-pumping ability of this pigment is retained (Marcus et al., 1977; Gärtner et al., 1983). In order to obtain information on chromophore and protein structural changes and on chromophore−protein interactions, the photocycle has been studied by FT-IR difference spectroscopy (Braiman & Rothschild, 1988; Siebert, 1990, 1993). Here, static methods are employed at 80, 170, and 213 K, stabilizing the intermediates K, L, and M. Steady-state experiments are performed to characterize the intermediate with the longest lifetime. Time-resolved techniques are applied to study the kinetics and dynamics of chromophore and protein: the late photocycle in the millisecond time range is investigated by the rapid-scan FT-IR method, whereas information on the kinetics of the M intermediate is obtained by conventional flash photolysis studies at 412 nm. The results are discussed with respect to the importance of the 9-methyl group for a functionally efficient chromophore−protein interaction. The data are consistently analyzed in terms of a sequential model of the BR photocycle that includes backreactions [e.g., Váró and Lanyi (1990)]. This does not mean that the results prove this model. Probably, the data could also be interpreted in a scheme with branched or parallel photocycles [e.g., Eisfeld et al., (1993)].

MATERIALS AND METHODS

The retinal analog was synthesized according to published schemes (Blatz et al., 1969; Waddel et al., 1978). The chromophore synthesized in the 9-*cis* form was converted to the *all-trans* isomer by irradiation with white light. Purple membranes were bleached and regenerated with the retinal analog as described (Tokunaga & Ebrey, 1978; Gerwert & Siebert, 1986). Spectroscopic measurements (IR and UV−vis) were performed on hydrated film samples (Engelhard et al., 1985). Before each experiment the sample was light-adapted by illumination with wavelengths above 495 nm for 5 min. In case of the low-temperature measurements, the sample was immediately cooled to the temperature of the experiment after light adaptation at room temperature. Prior to the time-resolved measurements, the sample was light-adapted at the temperature of the respective experiment.

Static low-temperature FT-IR difference spectra were obtained at 80, 170, and 213 K (Engelhard et al., 1985; Gerwert & Siebert, 1986). In order to increase the yield of M at 213 K, 1 μmol of borate buffer (pH 9.5) was added; otherwise no buffer was used. Steady-state measurements were performed at 278 K (Fahmy et al., 1992). Time-resolved FT-IR measurements were carried out with the rapid-scan technique (Kräutle et al., 1990; Fahmy et al., 1992). With this method, the first spectrum was measured 6 ms after laser excitation at a time resolution of approximately 11 ms and at a spectral resolution of 4 cm^{-1}. A total of 256 interferograms were averaged for measurements at 293 and 313 K. Only 6 interferograms were averaged at 263 K due to the long cycling time of the photocycle. The repetition rates of the laser pulses were 30 s, 4 s, and 5 min, respectively. For excitation of 9-demethyl-BR with its absorption maximum at 550 nm, a dye laser (520 nm, 4 mJ) pumped by an excimer laser was used. Native BR was excited at 530 nm. In some cases, to overcome the problem of low yield of photolysis using the short laser pulses (see Results), the sample was excited with flashes of 100-μs duration from a xenon flash lamp. A long-path filter (495 nm) and a heat-absorbing filter were inserted.

Time-resolved UV−vis transient absorption changes were measured with a conventional flash photolysis equipment using the same laser system for excitation. One hundred signals were averaged.

RESULTS

Static Low-Temperature FT-IR Difference Spectra. Figure 1 shows the static low-temperature FT-IR difference spectra of 9-H-BR^{550} at 80, 170, and 213 K. Under these conditions the respective BR → K, BR → L, and BR → M difference spectra of native BR^{568} are obtained (Siebert & Mäntele, 1983; Engelhard et al., 1985). A comparison of the 80 K spectrum from 9-H-BR^{550} (Figure 1a) with the native BR → K spectrum (Figure 2a) shows that the main spectral features are very similar. Particularly, the difference band of the ethylenic mode at 1534/1524 cm^{-1} indicates the appearance of a red-shifted intermediate. The upshift of the negative ethylenic mode from 1528 cm^{-1} in BR^{568} to 1534 cm^{-1} in 9-H-BR^{550} is due to the shift of the absorption maximum in the visible spectral range (Aton et al., 1977) from 568 nm (BR^{568}) to approximately 550 nm (9-H-BR^{550}). In order to determine the contribution of the 13-*cis*, 15-*syn* form of 9-H-BR (λ_{max} 530 nm; Marcus et al., 1977) to the 80 K spectrum, we measured the difference spectrum of the light/dark adaptation (data not shown): the absence of characteristic bands of the 13-*cis*, 15-*syn* form in Figure 1 shows that after light adaptation 9-H-BR^{550} contains essentially only *all-trans*-retinal. In the fingerprint region, the position of the C_{10}−C_{11} stretching vibration (Gerwert & Siebert, 1986; Smith et al., 1987) has shifted to 1146 cm^{-1}. The C_{14}−C_{15} stretching vibration is overlapped by the broad positive band at 1166 cm^{-1} representing both the C_{14}−C_{15} and C_{10}−C_{11} stretching

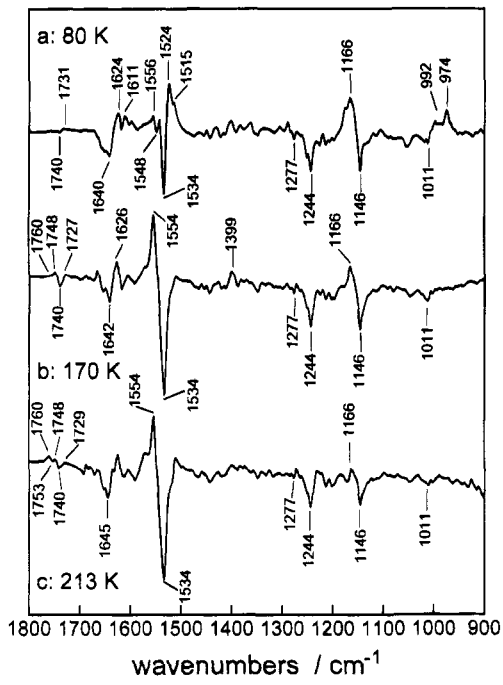

FIGURE 1: Low-temperature static difference spectra of 9-demethyl-BR taken at (a) 80 K, pH 7; (b) 170 K, pH 7; and (c) 213 K, pH 9.5. The last spectrum contains a mixture of L and M, indicating probably the presence of the M_1 state (see Discussion).

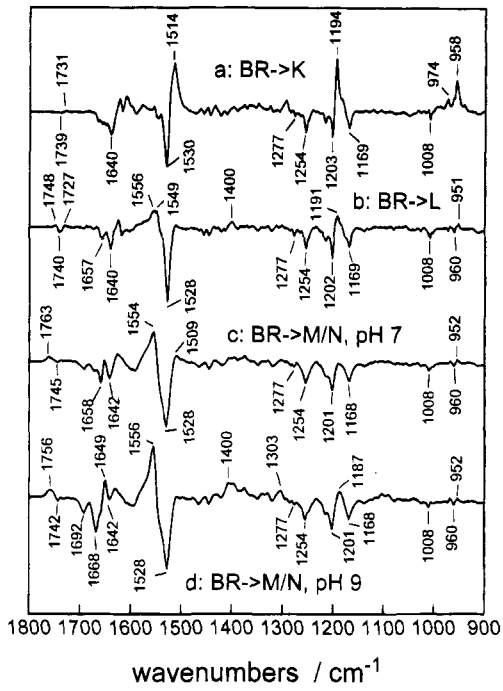

FIGURE 2: Static difference spectra of native BR: (a) BR → K taken at 80 K, pH 7; (b) BR → L taken at 170 K, pH 7; (c) steady-state BR → M/N taken at 268 K, pH 7; and (d) steady-state BR → M/N taken at 274 K, pH 9. pH 9 was selected in order to increase the amount of N.

modes of the photoproduct. The band corresponding to the delocalized mode observed for native BR^{568} at 1254 cm^{-1} (15-H, N−H, C_{14}−C_{15}) is now located at 1244 cm^{-1}. Probably due to the removal of the coupling with the rocking mode of the 9-methyl group, the fingerprint bands are downshifted as compared to native BR. They still indicate the *all-trans* to 13-*cis* isomerization of the chromophore. Remarkably, the position of the C=N stretching vibration

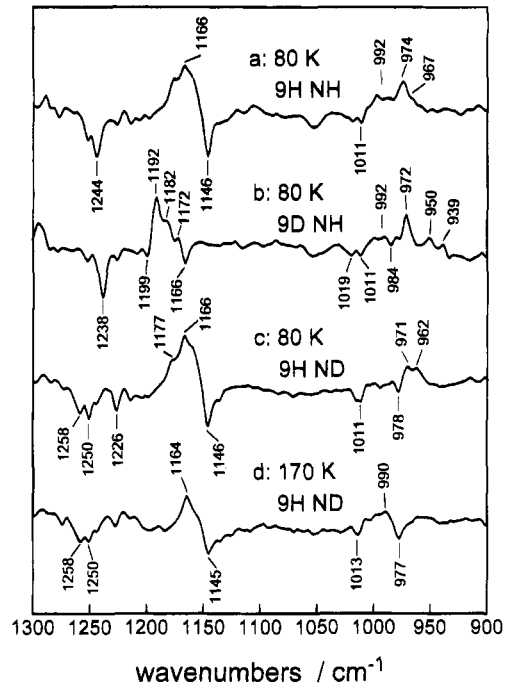

FIGURE 3: Low-temperature static difference spectra of 9-demethyl-BR in the spectral range between 1300 and 900 cm^{-1} showing the HOOP modes and fingerprint vibrations: (a) 9-H-BR^{550} in H_2O at 80 K; (b) 9-D-BR^{550} in H_2O at 80 K; (c) 9-H-BR^{550} in ^2H_2O at 80 K; and (d) 9-H-BR^{550} in ^2H_2O at 170 K.

of the protonated Schiff base in the initial state at approximately 1640 cm^{-1} has not changed. The 20-CH_3 bending vibration is upshifted by 3 cm^{-1} and absorbs now at 1011 cm^{-1}. As in native BR, the differential band due to protonated Asp115 undergoing environmental change in the BR → K transition (Braiman et al., 1988a) is located at 1740/1731 cm^{-1}, and the tyrosine mode of Tyr185 is seen at 1277 cm^{-1} (Braiman et al., 1988b). At 1515 cm^{-1}, a shoulder of the positive ethylenic mode is discernible; probably this band is also present in the BR → K spectrum of native BR (Figure 2a), but there it may be totally obscured by the ethylenic mode at 1514 cm^{-1}. This band may be caused by the C=C stretching mode of tyrosine since measurements on BR containing ring-deuterated tyrosine reveal in the BR → K spectrum of native BR a difference band at 1518/1512 (our unpublished results).

Important differences as compared to the spectrum of native BR are found in the spectral range of hydrogen out-of-plane (HOOP) bending vibrations (Figures 1a and 3a vs Figure 2a): a new broad feature appears at 992 cm^{-1}, and the band at 974 cm^{-1} has higher intensity and shows a shoulder at 967 cm^{-1}. The intense band at 958 cm^{-1} is missing. In order to assign these bands, the transition to the K-like photoproduct is measured for 9-^{2}H-BR^{550} (Figure 3b) and, in addition, for 9-H-BR^{550} in ^2H_2O (Figure 3c). The spectrum in Figure 3b shows the sensitivity of the C−C stretching modes upon deuteration of the retinal at C_9: the shape of the bands in the fingerprint region is altered. In the HOOP region drastic changes are observed: a negative band arises at 984 cm^{-1}, the large band at 974 cm^{-1} in Figure 3a has shifted to a new maximum at 972 cm^{-1} and is more narrow, and the shoulder at 967 cm^{-1} is absent. Instead, two new bands appear at 950 and 939 cm^{-1}. The interpretation of these changes is complicated: the disappearance of the coupled 9−10-HOOP mode, the appearance of the

isolated 10-HOOP mode and the appearance of the 9-^{2}H in-plane bending mode may contribute as negative and positive bands. From their positions, the new positive bands at 950 and 939 cm^{-1} can be assigned to the 9-^{2}H in-plane bending mode and to the 10-HOOP mode of the K-like intermediate (Colthup et al., 1975; Lin-Vien et al., 1991). The negative band at 984 cm^{-1} may be caused by the 9-^{2}H in-plane bending mode of the initial state. Since the small band at 967 cm^{-1} in Figure 3a is absent if the chromophore is deuterated at C_9 (Figure 3b), it appears plausible to assign it to the coupled 9−10-HOOP mode. The broad band at 992 cm^{-1} (Figures 1a and 3a) disappears in ^2H_2O (Figure 3c), and the band at 974 cm^{-1} is downshifted to 971 cm^{-1}. In addition, three new bands appear: at 962 cm^{-1} (positive), at 978 cm^{-1} (negative), and at approximately 975 cm^{-1} (positive). The last one is a weak, broad feature that can be inferred from the observation that the minimum of the negative band at 978 cm^{-1} is located above the baseline. The bands at 978 and 962 cm^{-1} are probably caused by the N^{2}H in-plane bending modes of the ground state and the K-like photoproduct, respectively. The broad band at 992 cm^{-1} disappears in ^2H_2O. It may be that the broad positive band at 975 cm^{-1} represents the corresponding mode in ^2H_2O. This would indicate that Schiff base deuteration causes a downshift of the broad band from 992 cm^{-1} to around 975 cm^{-1}. A similar behavior is observed for the 15-HOOP mode in the BR → KL spectrum of native BR at 983 cm^{-1} (Weidlich & Siebert, 1993). Therefore, the band at 992 cm^{-1} is assigned to the 15-HOOP mode, which is somewhat coupled to the NH-HOOP (Figures 1a and 3a). As a consequence, the band at 974 cm^{-1} in H_2O (Figure 3a) and 971 cm^{-1} in ^2H_2O (Figure 3c) cannot be caused by the 15-HOOP mode that is found in the BR → K spectrum of the native pigment at 958 cm^{-1} (Maeda et al., 1991). It could be caused by the 7−8-HOOP mode or the 11−12-HOOP mode, but no assignment can be made as yet.

In the spectrum of 9-H-BR^{550} taken at 170 K (Figure 1b), the characteristic features of the BR → L spectrum of native BR (Figure 2b) can be seen: the upshift of the ethylenic mode (to 1554 cm^{-1}), the reduced intensity of the C_{10}−C_{11} stretching mode (at 1166 cm^{-1}), and especially the band structure above 1700 cm^{-1} assigned to the C=O stretching frequencies of protonated Asp96 (1740/1748 cm^{-1}) and protonated Asp115 (1740/1727 cm^{-1}) (Braiman et al., 1988a; Gerwert et al., 1989; Sasaki et al., 1994). Deviations occur in the spectral range of HOOP vibrations below 1000 cm^{-1}: the small bands in the spectrum of native BR at 960 and 951 cm^{-1} (Figure 2b) are absent in the spectrum of the analog pigment (Figure 1b). The negative band at 960 cm^{-1} is assigned to the 11−12-HOOP mode by resonance Raman experiments (Smith et al., 1987). Also, the positive band at 951 cm^{-1} is probably caused by this HOOP mode because of its similar position. In the spectrum of the analog pigment obtained in ^2H_2O (Figure 3d), the typical band at 990 cm^{-1} can be seen that was assigned to the 15-HOOP mode (Stockburger et al., 1986). Its high intensity indicates a strong twist of the C_{14}−C_{15} single bond of the chromophore (Fahmy et al., 1989).

Complete conversion of native BR to M is obtained at 213 K if the pH is increased to 9.5 (Engelhard et al., 1985). In contrast, under these conditions the spectrum of 9-H-BR^{550} still exhibits some features of the L photoproduct, although with reduced intensity (Figure 1c). The C=O stretching

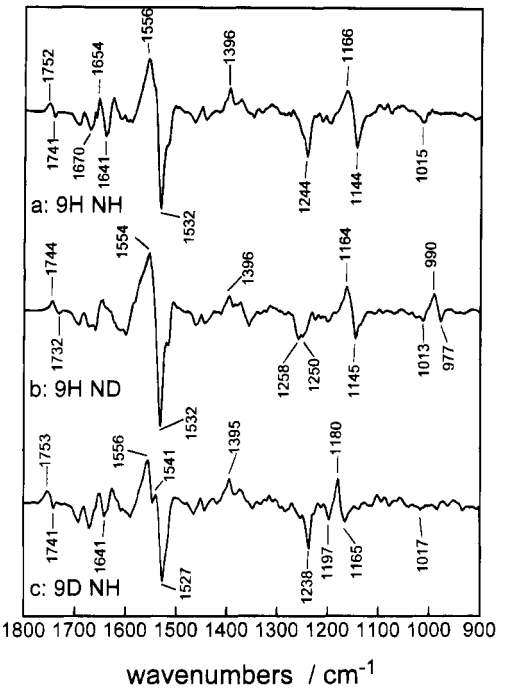

FIGURE 4: Static steady-state difference spectra of 9-demethyl-BR taken at 268 K and pH 7: (a) 9-H-BR^{550} in H_2O; (b) 9-H-BR^{550} in ^2H_2O; and (c) 9-D-BR^{550} in H_2O.

mode at 1760 cm^{-1}, due to protonation of Asp85 (Braiman et al., 1988a; Fahmy et al., 1992), indicates that some M has been formed (compare to Figure 2c). Consequently, the spectrum of 9-H-BR^{550} taken at 213 K (Figure 1c) contains a mixture of L and M intermediates.

Static Steady-State FT-IR Difference Spectra. Illumination of native BR at 268 K and pH 7 with continuous light produces a steady-state mixture of M and small amounts of N (Bousché et al., 1991; Fahmy et al., 1992; see also BR → M/N difference spectrum, Figure 2c). Figure 4a shows the difference spectrum of 9-H-BR^{550} taken under such conditions. This spectrum is clearly different from a BR → M spectrum but displays the typical features of a BR → N spectrum (Pfefferlé et al., 1991; Bousché et al., 1991; Hessling et al., 1993; Fahmy et al., 1993): the C=O stretching mode of protonated Asp85 (around 1755 cm^{-1}) is downshifted from its position in M, the negative band at 1742 cm^{-1} is due to deprotonated Asp96, pronounced amide I bands are present around 1668 cm^{-1} (negative) and 1653 cm^{-1} (positive), and a large positive amide II band at 1556 cm^{-1}, a positive band around 1396 cm^{-1} (NH bending of the protonated Schiff base coupled to $C_{15}H$ bending), and a positive fingerprint mode at 1166 cm^{-1} (C_{10}−C_{11} stretch) are observed. These features can also be seen in the steady-state BR → M/N spectrum taken at 274 K and pH 9 containing about 70% N (Weidlich & Siebert, 1993) (Figure 2d). As for the negative band of the initial state at 1144 cm^{-1}, the positive fingerprint band at 1166 cm^{-1} of N is downshifted as compared to native BR due to the lack of the 9-methyl group. Interestingly, the position of the band caused by Asp85 is slightly altered (1752 vs 1756 cm^{-1}), indicating an altered interaction of this group with its environment. As described for L (Maeda et al., 1991), the positive band at 1396 cm^{-1} is sensitive to $H_2O/^2H_2O$ exchange (Figure 4b) supporting the assignment to the N−H bending mode. In contrast to native BR (Figure 2d), the

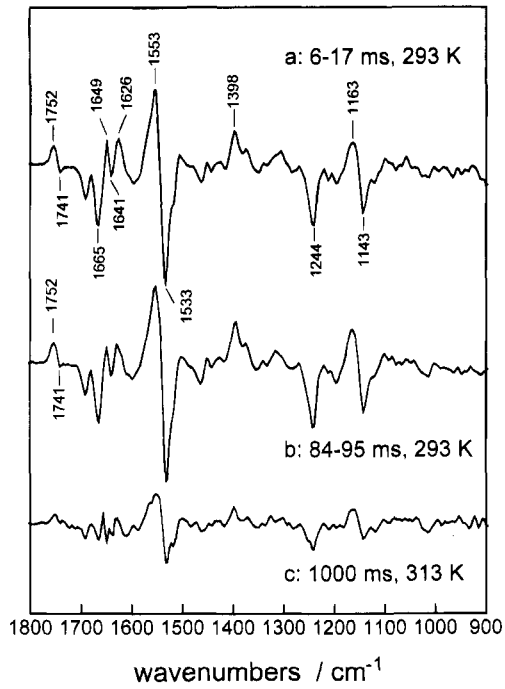

FIGURE 5: Time-resolved rapid-scan difference spectra of 9-demethyl-BR: (a) 6−17 ms after laser excitation taken at 293 K; (b) 84−95 ms after the flash taken at 293 K; and (c) 1000 ms after the flash taken at 313 K, showing the cycling time of approximately 1000 ms.

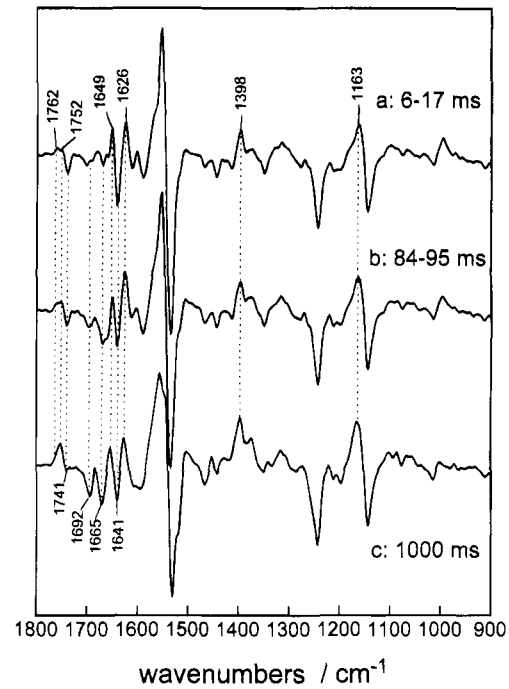

FIGURE 6: Time-resolved rapid-scan difference spectra of 9-demethyl-BR taken at 263 K: (a) 6−17 ms after the flash; (b) 84−95 ms after the flash; and (c) 1000 ms after the flash. Three N substates are distinguishable due to the size of the negative bands at 1665 and 1741 cm^{-1}.

N-like intermediate of 9-H-BR^{550} does not show any HOOP bands in the spectral range between 950 and 1000 cm^{-1} in H_2O. However in ^2H_2O, the 15-HOOP mode at 990 cm^{-1} appears, which is characteristic of the N intermediate with deuterated Schiff base (Figure 4b) (Weidlich & Siebert, 1993), and indicates a strong twist of the chromophore in its terminal part (Fahmy et al., 1991). The spectrum in Figure 4c taken under steady-state illumination shows the sensitivity of the negative ethylenic mode to deuteration of the retinal at C_9: it is downshifted to 1527 cm^{-1}. The major part of the positive band at 1556 cm^{-1} keeps its position and, therefore, is assigned to the amide II band. The shoulder at 1541 cm^{-1} corresponds to the shifted ethylenic mode of N. It can be concluded that, in contrast to native BR, little M accumulates under steady-state illumination at neutral pH, but mainly N is obtained. It should, however, be mentioned that the negative band at 1741 cm^{-1} due to deprotonated Asp96 has lower intensity than in N of native BR (Pfefferlé et al., 1991).

Time-Resolved Rapid-Scan FT-IR Difference Spectra. In performing time-resolved measurements, a peculiar observation was made: the extent of excited 9-H-BR^{550} strongly depends on the pulse width of the laser. At the same pulse energy (4 mJ) and wavelength (in this experiment 532 nm), the pulse of the ND·YAG laser with a duration of 5 ns excites less than 50% as compared to the pulse of the dye laser with a duration of 20 ns (data not shown). With the 20-ns pulse, the extent of photolysis is reduced about 5-fold in comparison to BR^{568}. This result is based on the amplitudes of a characteristic N band in the time-resolved FT-IR measurements of BR^{568} at pH 8.5 and 9-H-BR^{550}, i.e., the N−H bending mode at 1398 cm^{-1}. Figure 5 shows the time-resolved FT-IR difference spectra taken at 6−17 ms (a) and 84−95 ms (b) after laser excitation at 293 K. The spectrum taken at 6−17 ms is comparable to the spectrum obtained under steady-state conditions (Figure 4a) with the characteristics of the N intermediate. Since no positive band at 1760 cm^{-1}, due to protonated Asp85, is detectable, it is clear that no M is present in this time range. This is in contrast to the photocycle of native BR: at neutral pH only small amounts of N are observed and M is present during the lifetime of N. The bands in the later spectrum taken at 84−95 ms (Figure 5b) have approximately the same intensities, indicating that no decay has occurred. All spectral features are unchanged; only the negative band at 1741 cm^{-1} due to the C=O stretching mode of protonated Asp96 disappears, indicating reprotonation of Asp96. In contrast to normal BR, this occurs during the lifetime of N. In order to demonstrate the strongly prolonged cycling time of 9-H-BR, we measured the difference spectrum at 313 K and 1 s after the flash. The result is shown in Figure 5c. It is obvious that the spectrum shows the same features of the N intermediate. Only the amplitudes have decayed to about $^1/_3$ the original size. Thus, the cycling time is approximately 1 s at 313 K, at which temperature that of native BR is 4 ms only. Therefore, the cycling time of 9-H-BR is increased approximately 250-fold. It is remarkable that, as in native BR, the chromophore modes in N are very similar to those of L (Figure 1b). Even at the elevated temperature no O intermediate could be detected.

In order to obtain information on the M intermediate, which has obviously decayed in the time range so far monitored, we cooled the sample to 263 K. At this temperature, the cycling time of 9-H-BR amounts to 1 min. Thus, only after every 5 min could the sample be excited, which caused a total measuring time of 30 min if six spectra are averaged. The result is shown in Figure 6. Spectra a and b, taken in the time ranges of 6−17 and 84−95 ms, respectively, indicate that M is still present: the band at 1762 cm^{-1}, due to protonated Asp85 in the M state, is clearly

visible. In addition, the positive bands at 1398 and 1163 cm^{-1}, characteristic of N, have lower relative intensities than in the spectra of Figure 5a,b. The negative band at 1741 cm^{-1} is now larger, indicating a larger degree of deprotonation of Asp96 than in the same time range at 293 K. (It appears that the integral area of the positive bands at 1762 and 1752 cm^{-1} is smaller than that of the positive band at 1752 in spectrum c. This can be explained by the overlap with the negative band at 1741 cm^{-1}.) Furthermore, the negative amide I band at 1665 cm^{-1}, which for native BR is characteristic of the N state, has fully developed only in the spectrum taken at 1 s after the flash. At 293 K, this band is already present in full size in the first spectrum (Figure 5a). The positive amide I band at 1649 cm^{-1} clearly indicates the existence of N and excludes the contribution of L to the spectra in Figure 6. Thus, at least three N substates are distinguishable. The first one is characterized by the lack of fully developed negative amide I bands and by deprotonated Asp96 (Figure 6a). In the second one the amide I bands have developed but Asp96 is still deprotonated (Figure 6b). In the third N substate Asp96 is, in addition, reprotonated (Figures 5b and 6c).

We tried to obtain information on the KL, L, and M intermediates by the time-resolved step-scan FT-IR method (Uhmann et al., 1991; Weidlich & Siebert, 1993). However, the difficult measuring conditions such as the long cycling time and the low yield of photolysis impeded reliable measurements. In order to determine the rise time of M, we measured with conventional flash photolysis the absorbance changes at 412 nm. In addition to directly monitoring the M intermediate, this wavelength has the advantage that the monitoring beam does not evoke secondary reactions from the long-lived N interemediate. The results are shown in Figure 7 for 9-H-BR and native BR. In order to facilitate measuring conditions, the temperature was adjusted to 313 K. It is obvious that, as compared to M of native BR, M of 9-H-BR has a longer rise time (approximately 1.7-fold). Its decay is monophasic, in contrast to that of native BR. Similar results for the kinetics of the M intermediate of 9-H-BR are reported in a recent study (Yamazaki et al., 1995). A corresponding time course of M measured under conditions of Figure 6, i.e., at 263 K, shows that in this case the maximum M amplitude is obtained between 10 and 20 ms after the flash.

DISCUSSION

Our investigations of 9-demethylbacteriorhodopsin demonstrate that the removal of the 9-methyl group strongly changes the photocycle of BR: the time of the reaction cycle is slowed down about 250-fold, while the extent of photolysis is reduced about 5-fold and depends on the pulse width of the laser. The photoreaction passes through a slow-rising M intermediate and a very long-lived N intermediate. Three different N substates (N is defined by the characteristic chromophore modes) are distinguishable, as evidenced by the protonation state of Asp96 and by the size of the negative amide I band at 1665 cm^{-1}. The shift of 3 cm^{-1} of the amide I band to lower wavenumbers compared to the N intermediate of native BR indicates a slightly different conformation in the N substates of the analog pigment. Due to the long decay time of N, no O can be detected. The chromophore modes of the L and N intermediates are very similar. The intermediate obtained at 80 K exhibits a strong 15-HOOP

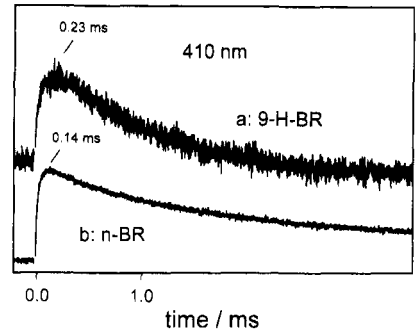

FIGURE 7: Flash-induced absorbance changes measured at 412 nm at 313 K: (A) 9-demethyl-BR and (b) native Br, showing the slower rise of M in the photocycle of 9-demethyl-BR. The much larger noise in trace a is due to the reduced photolysis yield.

mode. This is characteristic of the KL species (Weidlich & Siebert, 1993).

The last result is easily intelligible: the removal of the 9-methyl group decreases the steric interaction with the protein and allows the chromophore to relax already at 80 K to a KL-like intermediate. With respect to L formation, no special behavior can be deduced from the low-temperature difference spectrum. However, the difference spectrum measured at 213 K and high pH, conditions under which for native BR a complete conversion to M is achieved (Engelhard et al., 1985), shows that more than 20% L is present; i.e., the equilibrium is shifted toward the L state. In order to explain this result, several other observations have to be taken into consideration. The low-temperature M spectrum of native BR obtained under the same conditions (Engelhard et al., 1985) is very similar, also in the amide I range, to the M spectrum obtained from stroboscopic time-resolved FT-IR investigations and subsequent factor analysis (Hessling et al., 1993). If the general scheme as developed by Váró and Lanyi is accepted (i.e., a quasi-irreversible step between M_1 and M_2 and the L $\leftrightarrow$ M_1 equilibrium on the side of L; Váró & Lanyi, 1990, 1991), the similarity indicates that for native BR both the low-temperature M spectrum and the time-resolved M spectrum represent the M_2 state. There are now two possibilities to explain the presence of L in the low-temperature spectrum of 9-H-BR: either the quasi-irreversible step from M_1 to M_2 is blocked or the M_2 state is included into the equilibrium with L by increasing the backreaction from M_2 to M_1. In view of the result that at high pH the backreaction from M_2 to M_1 is decreased (Zimányi et al., 1992b), the first explanation seems to be more plausible. If this is correct, the spectrum shown in Figure 1c actually represents a BR $\rightarrow$ L/M_1 difference spectrum. Apparently, the removal of the 9-methyl group weakens the interaction of the chromophore with the protein in such a way that, at 213 K, the protein conformational changes involved with the formation of M_2 are inhibited.

The reduced yield of photolysis may be caused by an increased efficiency of the backreaction from the excited state, causing a very low yield of K (or KL) under photostationary-state conditions. This could also explain the higher yield for longer pulses: due to the increased efficiency for the backreaction a large amount of BR molecules return fast to the ground state, which are then available for additional excitations during the longer pulse. Alternatively, it could be that a thermal relaxation of K (or KL) takes place

during the longer pulse, which reduces the extent of backreaction if this intermediate is excited.

From the reproduced frequency of the C=N stetching vibration of the Schiff base of 9-H-BR^{550} (Figure 1), it can be concluded that the interaction of the NH group with the complex counterion (De Groot et al., 1990) has not changed. Therefore, the Schiff base is not displaced to a large extent despite the removal of the 9-methyl group. No clear positive or negative HOOP modes can be seen in the spectra of Figure 1b,c. This is in contrast to native BR, for which, in the L spectrum, negative and positive and, in the M spectrum, negative HOOP modes can be resolved (Figure 2). Apparently, the lack of the methyl group at C_9 allows the chromophore to adopt a more planar geometry in these states. Similar observations have been made for 9-H-rhodopsin (Ganter et al., 1989).

It is tempting to correlate the slow rise of M with the presence of L at 213 K. However, a few additional comments may be necessary. In the general scheme of the photoreaction as derived by Váró and Lanyi (1990, 1991), a slow rise of M can be due to a decrease of the L $\rightarrow$ M_1 rate constant, resulting in a reduced amount of M_1 in the equilibrium with L, or it can be caused by a decrease of the $M_1 \rightarrow M_2$ rate constant. Both interpretations appear to be plausible. In the first case, the removal of the 9-methyl group could alter the alignment of Asp85 with the Schiff base, thereby shifting the L/M_1 equilibrium toward the L state (altering the pK_a of the Schiff base). Such pK_a changes have recently been reported for mutations influencing the Schiff base region (Brown et al., 1994; Govindjee et al., 1995). For the second possibility one would extrapolate the results obtained at 213 K to the situation at room temperature; i.e., removal of the 9-methyl group influences the $M_1 \rightarrow M_2$ rate constant. Without more detailed kinetic analyses, one cannot decide between these two interpretations. In any case, the 9-H-BR system seems to offer the possibility to derive molecular differences between M_1 and M_2 using FT-IR spectroscopy (experiments in preparation).

It is the N state which is most strongly affected by removal of the methyl group. In the time-resolved spectra measured at 263 K (Figure 6), M coexists with N up to approximately 200 ms after the flash. However, in the spectrum taken 1 s after the flash (or at 293 K, 100 ms after the flash; Figure 5b), M has completely decayed. Thus, the several different forms of N and the long decay time interrupt the M $\leftrightarrow$ N equilibrium. In the first spectrum accessible to our measurements at 263 K (Figure 6a), the amide I bands differ from those observed at later times (e.g., negative band at 1665 cm^{-1}), although the fingerprint bands show all the characteristics of the N state. In the second spectrum (Figure 6b), amide I bands typical of the N state are now observed, whereas the amount of M has hardly changed, as can be deduced from the unchanged intensity of the C=O stretch of protonated Asp85 at 1762 cm^{-1}. (The apparent increase of the band at 1752 cm^{-1} is due to the decrease of the negative and at 1741 cm^{-1}, which has almost completely disappeared in the spectrum of Figure 6c.) Concomitantly with the decay of M, the fingerprint band at 1163 cm^{-1} increases in intensity by about 25% (Figure 6c). This shows that the decay of M is correlated with an increase in N. Therefore, the mixture of N and M in the spectrum of Figure 6a contains 75% N, although the negative amide I band at 1665 cm^{-1} has negligible intensity. This shows that the molecular changes mirrored by the formation of this amide I band occur for the largest part later than Schiff base reprotonation. This result is, at least partially, in variance with the interpretation of FT-IR data obtained for the Asp96 $\rightarrow$ Asn mutant at high pH. An intermediate "M_N" with deprotonated Schiff base but with N-like protein structure was identified and it was inferred that, also for native BR, the protein changes precede Schiff base reprotonation (Sasaki et al., 1992). In contrast, our data show that reprotonation of the Schiff base does not require those peptide backbone changes characteristic of the N state that cause the negative band at 1665 cm^{-1}. Apparently, these large amide I changes represent neither the molecular switch nor the conformational changes regulating the pK_a changes of Asp96 (Cao et al., 1993b; Rothschild et al., 1993). However, since the positive amide I band at 1649 cm^{-1}, also a characteristic feature of N, is already present in the spectrum of Figure 6a, the corresponding structural changes could be a feature of M_N and thus represent a prerequisite for Schiff base reprotonation from Asp96. Since, as it was stated in Results, at 263 K the maximum M formation occurs around 15 ms after the flash, the apparent amplitude of M amounts to 25% only. This can be explained by an efficient M $\rightarrow$ N_1 transition that reduces the apparent M amplitude.

In the early spectra, Asp96 is fully deprotonated. It reprotonates later, without changes of the chromophore bands taking place. From the spectra in Figure 6 one could argue that the disappearance of the band at 1741 cm^{-1} is correlated with the M decay, as evidence by the disappearance of the positive band at 1762 cm^{-1}. However, the lack of the band at 1762 cm^{-1} in Figure 5a demonstrates that M has already completely decayed, whereas the remaining negative band at 1741 cm^{-1} indicates that there is still some fraction of Asp96 deprotonated. Thus, as for native BR, it is not possible to correlate the M decay with the reprotonation of Asp96. It would be interesting to see whether the kinetics of reprotonation of Asp96 correlate with the proton uptake. Such experiments are in preparation. In any case, the results obtained for N show that removal of the 9-methyl group decouples chromophore reactions from protein reactions. In native BR at neutral pH, reprotonation of Asp96 is suggested to be correlated with the N $\rightarrow$ O transition, i.e., with the 13-*cis* $\rightarrow$ *all-trans* backisomerization (Souvignier & Gerwert, 1992; Bousché et al., 1992). Since this isomerization is slowed down 250-fold in 9-H-BR, reprotonation of Asp96 occurs earlier. An N state in which a proton was already taken up (by Asp96 or another group) has been postulated for native BR at high pH (Ames & Mathies, 1992; Cao et al., 1993a; Zimányi et al., 1993). The growing of the negative amide I band around 1665 cm^{-1} in N may indicate a conformational change of the protein that is a prerequisite for the thermal isomerization. All three N states of the pigment analog are characterized by the same strong 15-HOOP mode if the Schiff base is deuterated, comparable to the 15-HOOP mode observed for the L intermediate. Therefore, the basic geometry of the chromophore is the same in the three states and very similar to that in L.

The question arises which molecular mechanism causes this dramatic slowing down of the N decay. We propose the following picture: the lack of the methyl group enables the chromophore to adopt a large number of conformers in N which differ in twists around single bonds. In order to achieve the backisomerization, however, a special geometry

has to be adopted in the transition state. Thus, the barrier for the transition is increased by a corresponding reduction of the entropy. In native BR, the protein−chromophore interaction, mediated by the 9-methyl group, prepares the chromophore in N already in a conformation favorable for isomerization. To test this hypothesis, a more detailed kinetic analysis of time-resolved UV−vis absorbance changes is required. Such experiments will be published elsewhere (J. K. Lanyi and F. Siebert, manuscript in preparation). It is an interesting question which part of the protein interacts with the methyl group. From the model derived by Henderson, Trp182 is the amino acid with the closest contact (Henderson et al., 1990). Indeed, recent FT-IR observations on the L intermediate of the Trp182 → Phe mutant indicate that the NH group of Trp182 undergoes a change in hydrogen bonding, which is not observed in 9-H-BR. Additionally, the mutant exhibits delayed M formation. In this respect it is comparable to 9-H-BR (Yamazaki et al., 1995). These findings have been interpreted in terms of an interaction of the 9-methyl group of the retinal with Trp182 during the L → M transition. Our own results indicate that, especially in N, Trp182 undergoes molecular changes which are affected by the removal of the 9-methyl group (J. K. Lanyi, O. Weidlich, and F. Siebert, manuscript in preparation). The molecular model shows that the chromophore is tightly packed between Trp86 and Trp182, which provides an effective coupling of chromophore and protein molecular changes, required for the precisely tuned pumping mechanism. Finally, a comment should be made on the apparent normal pumping efficiency of 9-H-BR (Marcus et al., 1977; Gärtner et al., 1983). One would expect that for such a slow photocycle the efficiency would be drastically reduced under conditions of continuous illumination. It could be that in those experiments the proton transport was not limited by photocycle kinetics but by the light intensity. However, since the photocycle saturates at very low light intensities due to the accumulation of the long-lived N intermediate, we regard this as unlikely. Therefore, we present the following explanation: preliminary UV−vis photolysis experiments showed that the long-lived N state is easily excited by the visible probing beam. Thus it is possible that the photocycle is shortened by simultaneous irradiation of the N state, which increases the pumping efficiency under these conditions.

ACKNOWLEDGMENT

Stimulating discussions with J. K. Lanyi are gratefully acknowledged. We thank A. Maeda for informing us about his investigations on 9-H-BR prior to publication.

REFERENCES

Ames, J. B., & Mathies, R. A. (1990) *Biochemistry 29*, 7181−7190.

Aton, B., Doukas, A., Callender, R. H., Becher, B., & Ebrey, T. G. (1977) *Biochemistry 16*, 2995−2999.

Blatz, P. E., Lin, M., Balasubramaniyan, P., Balasubramaniyan, V., & Dewhurst, P B. (1969) *J. Am. Chem. Soc. 91*, 5930−5931.

Bousché, O., Braiman, M. S., He, Y.-W., Marti, T., Khorana, H. G., & Rothschild, K. J. (1991), *J. Biol. Chem. 266*, 11063−11067.

Bousché, O., Sonar, S., Krebs, M. P., Khorana, H. G., & Rothschild, K. J. (1992) *Photochem. Photobiol. 56*, 1085−1092.

Brack, T. L., Gärtner, W., & Atkinson, G. H. (1992) *Chem. Phys. Lett. 190*, 298−304.

Brack, T. L., Delaney, J. K., Atkinson, G. H., Albeck, A., Sheves, M., & Ottolenghi, M. (1993) *Biophys. J. 65*, 964−972.

Braiman, M. S. & Mathies, R. A. (1980) *Biochemistry 19*, 5421−5428.

Braiman, M. S., & Mathies, R. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 403−407.

Braiman, M. S., & Rothschild, K. J. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 541−570.

Braiman, M. S., Mogi, T., Marti, T., Stern, L. J., Khorana, H. G., & Rothschild, K. J. (1988a) *Biochemistry 27*, 8516−8520.

Braiman, M. S., Mogi, T., Stern, L. J., Hackett, N. R., Chao, B. H., Khorana, H. G., & Rothschild, K. J. (1988b) *Proteins: Struct., Funct., Genet. 3*, 219−229.

Brown, L. S., Gat, Y., Sheves, M., Yamzaki, Y., Maeda, A., Needleman, R., & Lanyi, J. K. (1994) *Biochemistry 33*, 12001−12011.

Cao, Y., Brown, L. S., Needleman, R., & Lanyi, J. K. (1993a) *Biochemistry 32*, 10239−10248.

Cao, Y., Váró, G., Klinger, A. L., Czajkowski, D. M., Braiman, M. S., Needleman, R., & Lanyi, J. K. (1993b) *Biochemistry 32*, 1981−1990.

Colthup, N. B., Daly, L. H., & Wiberley, S. E. (1975) in *Introduction to infrared and Raman spectroscopy*, Academic Press, New York.

De Groot, H. J. M., Smith, S. O., Courtin, J., Van den Berg, E., Winkel, C., Lugtenburg, J., Griffin, R. G., & Herzfeld, J. (1990) *Biochemistry 29*, 6873−6883.

Dencher, N. A., Dresselhaus, D., Zaccai, G., & Büldt, G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7876−7879.

Druckmann, S., Friedman, N., Lanyi, J. K., Needleman, R., Ottolenghi, M., & Sheves, M. (1992) *Photochem. Photobiol. 24*, 1041−1047.

Ebrey, T. G. (1993) in *Thermodynamics of membranes, receptors and channels*, CRC Press, Boca Raton, FL.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400−407.

Eisfeld, W., Pusch, C., Diller, R., Lohrmann, R., & Stockburger, M. (1993), *Biochemistry 32*, 7196−7215.

Fahmy, K., Grossjean, M. F., Siebert, F., & Tavan, P. (1989) *J. Mol. Struct. 214*, 257−288.

Fahmy, K., Siebert, F., & Tavan, P. (1991) *Biophys. J. 60*, 989−1001.

Fahmy, K., Weidlich, O., Engelhard, M., Tittor, J., Oesterhelt, D., & Siebert, F. (1992) *Photochem. Photobiol. 56*, 1073−1083.

Fahmy, K., Weidlich, O., Engelhard, M., Sigrist, H., & Siebert, F. (1993) *Biochemistry 32*, 5862−5869.

Fendler, K., Gärtner, W., Oesterhelt, D., & Bamberg, E. (1987) *Biochim. Biophys. Acta 893*, 60−68.

Gärtner, W., Towner, P., Hopf, H., & Oesterhelt, D. (1983) *Biochemistry 22*, 2637−2644.

Ganter, U. M., Schmid, E. D., Perez-Sala, D., Rando, R. R., & Siebert, F. (1989) *Biochemistry 28*, 5954−5962.

Gerwert, K., & Siebert, F. (1986) *EMBO J. 5*, 805−811.

Gerwert, K., Hess, B., Soppa, J., & Oesterhelt, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4943−4947.

Govindjee, R., Dancshazy, Z., Ebrey, T. G., Longstaff, C., & Rando, R. R. (1988) *Biophys. J. 54*, 557−562.

Govindjee, R., Kono, M., Balashov, S. P., Imasheva, E., Sheves, M., & Ebrey, T. G. (1995) *Biochemistry 34*, 4828−4838.

Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., Beckmann, E., & Downing, K. H. (1990) *J. Mol. Biol. 213*, 899−929.

Hessling, B., Souvignier, G., & Gerwert, K. (1993) *Biophys. J. 65*, 1929−1941.

Khorana, H. G. (1988), *J. Biol. Chem. 263*, 7339−7442.

Kräutle, R., Gärtner, W., Ganter, U. M., Longstaff, C., Rando, R. R., & Siebert, F. (1990) *Biochemistry 29*, 3915−3923.

Lanyi, J. K. (1992) *J. Bioenerg. Biomembr. 24*, 169−179.

Lanyi, J. K. (1993), *Biochim. Biophys. Acta 1183*, 241−261.

Lin-Vien, D., Colthup, N. B., Fateley, W. G., & Grasselli, J. G. (1991) in *The handbook of infrared and Raman characteristic frequencies of organic molecules*, Academic Press, Boston, MA.

Longstaff, C., & Rando, R. R. (1987) *Biochemistry 26*, 6107−6113.

Maeda, A., Sasaki, J., Pfefferlé, J. M., Shichida, Y., & Yoshizawa, T. (1991) *Photochem. Photobiol. 54*, 911−921.

Maeda, A., Sasaki, J., Shichida, Y., Yoshizawa, T., Chang, M., Ni, B. F., Needleman, R., & Lanyi, J. K. (1992) *Biochemistry 31*, 4684−4690.
Marcus, M. A., & Lewis, A. (1977) *Science 195*, 1328−1330.
Marcus, M. A., Lewis, A., Racker, E., & Crespi, H. (1977) *Biochem. Biophys. Res. Commun. 78*, 669−675.
Nakasako, M., Kataoka, M., Amemiya, Y., & Tokunaga, F. (1991) *FEBS Lett. 292*, 73−75.
Noguchi, T., Kolaczkowski, S., Gärtner, W., & Atkinson, G. H. (1990) *J. Phys. Chem. 94*, 4920−4926.
Oesterhelt, D., Tittor, J., & Bamberg, E. (1992) *J. Bioenerg. Biomembr. 24*, 181−191.
Pfefferlé, J. M., Maeda, A., Sasaki, J., & Yoshizawa, T. (1991) *Biochemistry 30*, 6548−6556.
Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.
Rothschild, K. J., Marti, T., Sonar, S., He, Y.-W., Rath, P., Fischer, W. B., & Khorana, H. G. (1993) *J. Biol. Chem. 268*, 27046−27052.
Sasaki, J., Shichida, Y., Lanyi, J. K., & Maeda, A. (1992), *J. Biol. Chem. 267*, 20782−20786.
Sasaki, J., Lanyi, J. K., Needleman, R., Yoshizawa, T., & Maeda, A. (1994) *Biochemistry 33*, 3178−3184.
Schiffmiller, R., Callender, R. H., Waddell, W. H., Govindjee, R., Ebrey, T. G., Kakitani, H., Honig, B. H., & Nakanishi, K. (1985) *Photochem. Photobiol. 41*, 563−567.
Siebert, F. (1990) in *Methods in Enzymology: Retinoids* (Packer, L., Ed.) pp 123−136, Academic Press, Inc., Orlando, FL.
Siebert, F. (1993) in *Biomolecular Spectroscopy, Part A* (Clark, R. J. H., & Hester, R. E., Eds.) pp 1−54, John Wiley & Sons, Chichester, U.K.
Siebert, F., & Mäntele, W. (1983) *Eur. J. Biochem. 130*, 565−573.
Smith, S. O., Pardoen, J. A., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. A. (1983) *Biochemistry 22*, 6141−6148.
Smith, S. O., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1987), *J. Phys. Chem. 91*, 804−819.
Souvignier, G., & Gerwert, K. (1992) *Biophys. J. 63*, 1393−1405.
Stockburger, M., Alshuth, T., Oesterhelt, D., & Gärtner, W. (1986) in *Spectroscopy of Biological Systems* (Clark, J. H., & Hester, R. E., Eds.) pp 483−535, Wiley & Sons, Chichester, U.K.
Subramaniam, S., Gerstein, M., Oesterhelt, D., & Henderson, R. (1993) *EMBO J. 12*, 1−8.
Tokunaga, F., & Ebrey, T. G. (1978) *Biochemistry 17*, 1915−1922.
Tsuda, M., Glaccum, M., Nelson, B., & Ebrey, T. G. (1980) *Nature 287*, 351−353.
Uhmann, W., Becker, A., Taran, C., & Siebert, F. (1991) *Appl. Spectrosc. 45*, 390−397.
Váró, G., Zimányi, L., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992) *Biophys. J. 61*, 820−826.
Váró, G., & Lanyi, J. K. (1990) *Biochemistry 29*, 2241−2250.
Váró, G., & Lanyi, J. K. (1991) *Biochemistry 30*, 5008−5015.
Waddel, W. H., Umerus, M., & West, J. L. (1978) *Tetrahedron Lett. 35*, 3223−3226.
Weidlich, O., & Siebert, F. (1993) *Appl. Spectrosc. 47*, 1394−1400.
Yamazaki, Y., Sasaki, J., Hatanaka, M., Kandori, H., Maeda, A., Needleman, R., Shinada, T., Yoshihara, K., Brown, L. S., & Lanyi, J. K. (1995) *Biochemistry 34*, 577−582.
Zimányi, L., Cao, Y., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992a) *Photochem. Photobiol. 56*, 1049−1055.
Zimányi, L., Váró, G., Chang, M., Ni, B., Needleman, R., & Lanyi, J. K. (1992b) *Biochemistry 31*, 8535−8543.
Zimányi, L., Cao, Y., Needleman, R., Ottolenghi, M., & Lanyi, J. K. (1993), *Biochemistry 32*, 7669−7678.
Zinth, W., Dobler, J., Franz, M. A., & Kaiser, W. (1988) in *Spectroscopy of Biological Molecules—New Advances Schmid*, E. D., Schneider, F. W., & Siebert, F., Eds.) pp 269−274, John Wiley & Sons, Chichester, U.K.

BI950065S