infrared spectrophometry, N.R.C.C. Bulletin No. 18, Ottawa, Canada.
Paterson, Y., Rumsey, S. M., Benedetti, E., Nemethy, G., & Sheraga, H. A. (1981) *J. Am. Chem. Soc. 103*, 2947-2955.
Pavone, V., Di Blasio, B., Santini, A., Benedetti, E., Pedone, C., Toniolo, C., & Crisma, M. (1990) *J. Mol. Biol. 214*, 633-635.
Pavone, V., Benedetti, E., Di Blasio, B., Pedone, C., Santini, A., Bavoso, A., Toniolo, C., Crisma, M., & Sartore, L. (1990) *J. Biomol. Struct. Dyn. 7*, 1321-1331.
Pletnev, V. Z., Gromov, E. P., & Popov, E. M. (1973) *Khim. Prir. Soedin. 9*, 224-229.
Popot, J. L., Engelman, D. M., Gurel, O., & Zaccai, G. (1990) *J. Mol. Biol. 210*, 829-847.
Prasad, B. V. V., & Balaram, P. (1984) *CRC Crit. Rev. Biochem. 16*, 307-348.
Pulla Rao, C., Nagaraj, R., Rao, C. N. R., & Balaram, P. (1980) *Biochemistry 19*, 425-431.
Reddy, G. L., & Nagaraj, R. (1989) *J. Biol. Chem. 264*, 16591-16597.
Rizzo, V., Stankowski, S., & Schwarz, G. (1987) *Biochemistry 26*, 2751-2759.
Rosenblatt, M., Beaudette, N. V., & Fasman, G. D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3983-3987.
Saitô, H., Tabeta, R., Formaggio, F., Crisma, M., & Toniolo, C. (1988) *Biopolymers 27*, 1607-1617.
Shinnar, A. E., & Kaiser, E. T. (1984) *J. Am. Chem. Soc. 106*, 5006-5007.
Smith, G. D., Pletnev, V. Z., Duax, W. L., Balasabramanian, T. M., Bosshard, H. E., Czerwinski, E. W., Kendrick, N. E., Matthews, F. S., & Marshall, G. R. (1981) *J. Am. Chem. Soc. 103*, 1493-1501.
Toniolo, C. (1989) *Biopolymers 28*, 247-257.
Toniolo, C., & Benedetti, E. (1988) *ISI Atlas Sci.: Biochem. 1*, 225-230.
Toniolo, C., Bonora, G. M., Barone, V., Bavoso, A., Benedetti, E., Di Blasio, B., Grimaldi, P., Lelj, F., Pavone, V., & Pedone, C. (1985) *Macromolecules 18*, 895-902.
Toniolo, C., Bonora, G. M., Bavoso, A., Benedetti, E., Di Blasio, B., Pavone, V., & Pedone, C. (1986) *Macromolecules 19*, 472-479.
Venkatachalapathi, Y. V., & Balaram, P. (1981) *Biopolymers 20*, 1137-1145.

Fourier Transform Infrared Study of the N Intermediate of Bacteriorhodopsin†

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ABSTRACT: Visible absorption spectroscopic experiments show that the N intermediate is the main photoproduct of a highly hydrated film of the light-adapted bacteriorhodopsin (70% water by weight) at pH 10 and 274 K. The difference Fourier transform infrared spectrum between the N intermediate and unphotolyzed light-adapted bacteriorhodopsin was recorded under these conditions. A small amount of the M intermediate present did not affect this spectrum significantly. The difference spectrum exhibited a positive band at 1755 cm^{-1} (probably due to Asp-85) and a negative band at 1742 cm^{-1} (due to Asp-96), neither of which was observed for the M intermediate. The spectrum of the N intermediate at pH 7 was nearly identical with that at pH 10. Spectra at pH 10 also were measured with isotope-substituted samples. A vibrational band at 1692 cm^{-1} due to the peptide bond disappeared, and a band at 1558 cm^{-1} emerged upon formation of the N intermediate. The spectrum also displayed bands containing the N-H and C_{15}-H in-plane bending vibrational modes at 1394 and 1303 cm^{-1}. These frequencies are similar to those of the L intermediate while the intensities of these bands are larger than those in the L intermediate, suggesting that the Schiff bases of both the L and N intermediates have a strong hydrogen-bonding interaction with the protein and that the C_{12}-H to C_{15}-H region of the chromophore is less twisted in the N intermediate than in the L intermediate.

Bacteriorhodopsin in the purple membrane (bR)[1] is one of the retinoid proteins produced by *Halobacterium halobium* (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1982). Its chromophore is retinal, linked to the protein moiety through the lysine-216 residue as a protonated Schiff base. Upon absorption of light by the chromophore, bR acts as a light-driven proton pump, by ejecting protons from the cell and taking up protons from the opposite side of the membrane. Asp-85 and -96, buried in the membrane, have been shown to be essential for these processes (Mogi et al., 1988), Asp-85 for the proton release step (Otto et al., 1990) and Asp-96 for the proton uptake step (Holz et al., 1989).

Resonance Raman experiments by Maeda et al. (1986) have revealed a photoproduct, L′, which is more stable at alkaline pH. By means of visible absorption spectroscopy, Drachev et al. (1987), Dancshazy et al. (1988), and Kouyama et al. (1988)

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[1] Abbreviations: bR, bacteriorhodopsin; FT-IR, Fourier transform infrared; BR^L, light-adapted bacteriorhodopsin; λ_{max}, wavelength of maximum absorption.

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have then observed a similar photoproduct called P, R, and N, respectively, whose decay is closely related with the proton uptake step. Time-resolved resonance Raman studies by Fodor et al. (1988a) have shown that N is produced by the decay of M. In extensive time-resolved studies, an equilibrium between M and "L" (Alshuth & Stockburger, 1986) or N (Ames et al., 1990; Vârô & Lanyi, 1990a) has been proposed. Later, L′ was shown to be the same as N (Nakagawa et al., 1990). A similar photoproduct, L′(B), has also been noticed in parallel with M (Diller & Stockburger, 1988). In view of the position in the photocycle and spectral properties, these intermediates are probably the same. The name of N will be used in this paper.

Fourier transform infrared (FT-IR)[1] spectroscopic studies have elucidated the protonation states of aspartic acid residues in K, L, and M intermediates that appear during the early stages of the photocycle (Engelhard et al., 1985; Braiman et al., 1988). In unphotolyzed *all-trans*-bR, Asp-85 and -96 are in the unprotonated and protonated states, respectively (Braiman et al., 1988; Gerwert et al., 1989). Asp-96 exhibits a large change upon the formation of L as a result of its deprotonation (Braiman et al., 1988) or of H-bonding changes (Gerwert et al., 1989). These changes continue into the time range of M. Asp-85 becomes protonated at the M stage by accepting the proton from the Schiff base (Braiman et al., 1988). The Asp-96 → Asn mutation slows down the decay of M remarkably, suggesting that Asp-96 is involved in the proton donation to the Schiff base of M (Holz et al., 1989; Otto et al., 1989).

The aim of the present work was to record the FT-IR spectrum on the N intermediate. The vibrational bands of the carboxylic acids, the peptide bonds, and the Schiff base were examined to compare with those of other intermediates, and the results were discussed in relation to the proton translocation pathway.

Materials and Methods

bR. The preparation of bR in purple membrane suspensions (A_{570nm} = 5) from *Halobacterium halobium* (strain ET1001; donated by Dr. T. Kouyama) has been described by Oesterhelt and Stoeckenius (1974). C_{15}-Deuteriated bR was prepared by adding C_{15}-deuteriated retinal to a culture medium for growing *H. halobium* in the presence of 1.0 mM nicotine (Spudich & Stoeckenius, 1980; Siebert & Mäntele, 1983). The extent of the in vivo synthesis of bR was checked by using the cell lysate as described by Danon et al. (1974). ϵ-^{15}N-Labeled bR ([^{15}N]bR) was made as described by Argade et al. (1981): [ϵ-^{15}N]lysine (MSD Isotopes, Montreal, Quebec, Canada) was added to a synthetic medium devoid of lysine. For calculation of the amount of bR, a molar extinction coefficient for the light-adapted bR of 63 000 (Oesterhelt & Hess, 1973) was used.

Sample Manipulation for FT-IR Measurements. For experiments with bR at pH 7, about 0.2 mg of bR suspended in 0.08–0.10 mL of water was deposited on a BaF_2 window (16 mm in diameter) and then dried in a Toshiba DC-47 drybox at 30% humidity atmosphere. For bR at pH 10, 0.4 mg of a bR suspension was mixed with 2.5 mL of 0.02 M borate buffer (pH 10). After centrifugation at 15 000 rpm for 30 min in a TMA-4 rotor of a Tomy high-speed microrefrigerated MRX-150 centrifuge at 4 °C, the pellet was resuspended in 2.5 mL of the same buffer, and the centrifugation was repeated. The final pellet was suspended in 0.15 mL of the buffer. Half of the suspension was deposited on the window and then dried at 4 °C over $CaCl_2$ for 5 h. All these procedures after mixing with pH 10 buffer were performed in the dark. The stability of bR during these procedures was checked by dissolving the dried bR in 0.005 M phosphate buffer (pH 7) and comparing the absorbance at 570 nm before and after drying at pH 10. The difference between them was less than 4%, indicating no denaturation during sample preparation.

A humidified sample was prepared as follows: the dried film of bR was humidified by placing 0.001 mL of water or heavy water (99.75%; Wako Pure Chemical Industries, Osaka, Japan) in a small hole in a silicone O-ring, prior to sealing the film with another BaF_2 window. A highly hydrated sample was prepared by humidifying the dried film with about 20 tiny droplets of water (or heavy water) on the surface of the dried film, followed by placing another 0.002 mL of water (or heavy water) in the hole of the O-ring before sealing it. On the basis of the report of Braiman et al. (1987), the water content of the samples was roughly estimated from the intensity around 3400 cm^{-1} to be about 50% by weight for the conventional humidified bR and about 70% by weight for the highly hydrated bR. All the manipulations with heavy water were done in a glovebag under an atmosphere of dry argon (Atmos bag, Aldrich) until the sealing.

Irradiation. The light source was a 1-kW tungsten lamp in a slide projector with a 5-cm water filter to remove heat. The wavelength for irradiation of the sample was selected by inserting a cutoff filter (Toshiba). The sample was first converted to the light-adapted state (BR^L)[1] by 5-min illumination at 274 K with light of wavelength longer than 470 nm (>470-nm light), and then the temperature desired was set. For the experiments at 274 K, irradiation was done just after light-adaptation. At 274 or 260 K, irradiation was done for 20 s to avoid heating the sample. After BR^L was irradiated at 230 K for 5 min with light of wavelength longer than 500 nm (>500-nm light), it was warmed to 274 K to ensure complete thermal decay before repetition of the same photoreaction at 230 K. The L/BR^L spectrum was obtained by irradiating BR at 170 K for 10 min with light of wavelength longer than 600 nm (>600-nm light). Subsequently, warming to 274 K, cooling, irradiation, and the measurements repeated 4 times.

FT-IR Measurements. The sample cell was mounted on an Oxford DN-1754 vacuum cryostat connected to an Oxford ITC 4 temperature controller, which maintained the temperature to within 0.1 K. The air in the cryostat was evacuated to 10^{-5} torr. FT-IR spectra were recorded in a Nicolet 60-SX spectrometer before and from 2 s after the irradiation unless otherwise specified. The spectral difference before and after irradiation was calculated. The spectrum of a long-lived species, M at 230 K, was obtained by summing 1024 interferograms, which required about 8 min. The N and M intermediates are short-lived at 274 and 260 K, so without increasing the temperature a cycle of irradiation and summation of 128 interferograms within 1 min was repeated 30 times to get the final spectra.

The spectra are depicted by adjusting the intensity of the negative band at 1202 cm^{-1} to the same height in the figures when comparisons were made between the M/BR^L and N/BR^L spectra (Figures 3–5) or between the spectra of isotope-substituted bR (Figures 7–9) except for C_{15}-deuteriated bR (Figures 7b and 8b). Since the 1202 cm^{-1} band is absent in the spectrum of the C_{15}-deuteriated BR^L [not shown in figures; see Siebert and Mäntele (1983)], the 1167 cm^{-1} band was used for normalization. The short horizontal line on the ordinate through Figures 3–9 represents zero absorbance.

Measurements of the Absorption Spectrum in the Visible Region. To measure the visible absorption spectrum, a highly

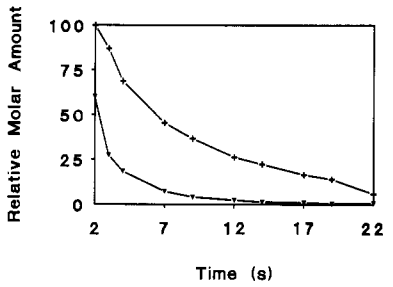

FIGURE 1: Time-dependent changes in the relative molar amount of M (▼) and N (+) for a highly hydrated film of BR^L at pH 10 at 274 K after irradiation with >470-nm light for 20 s. The molar amounts of M and N were calculated as described in the text. The ordinate represents the relative molar amount of M or N to the molar amount of N present at 2 s.

hydrated film of bR on a small BaF_2 window (10 mm in diameter) was used. The sample was mounted on an Oxford helium CF-1204 cryostat. The temperature was maintained within 0.1 K with an Oxford ITC 4 temperature controller. From 2 s after the irradiation of BR^L with >470-nm light, the absorbance changes at fixed wavelengths were recorded with Shimadzu MPS-2000 spectrophotometer at intervals of 2 or 3 s.

RESULTS

Difference Absorption Spectrum in the Visible Region. In order to obtain the difference spectrum of N to BR^L, experimental conditions under which a sufficient amount of N is present have to be found. Since it is known that alkaline conditions increase the stability of N (Maeda et al., 1986; Ames & Mathies, 1990; Váró & Lanyi, 1990a) and decrease the amount of O (Li et al., 1984), and that lowering the hydration increases the stability of M (Korenstein & Hess, 1977; Váró & Lanyi, 1991), the highly hydrated bR film with 70% water by weight was used. It was first irradiated at 274 K with >470-nm light for 5 min to light-adapt the sample.

Time-dependent absorbance changes at 570 and 410 nm were measured at intervals of 2 or 3 s from 2 to 62 s after 20-s irradiation of BR^L at the same temperature. The increase in absorbance at 410 nm largely reflects the amount of M [the wavelength for maximum absorption (λ_{max})[1] around 410 nm (Lozier et al., 1975)], and the decrease in absorbance at 570 nm resulted from the conversion of BR^L to both M and N. N has its λ_{max} around 550–570 nm with a lower extinction than BR^L, whose λ_{max} is at 568 nm (Drachev et al., 1987; Kouyama et al., 1988; Váró et al., 1990b). The relative molar amounts of M and N to that of N at 2 s were calculated from the decrease in the absorbance at 410 nm and the increase at 570 nm by assuming the molar increases of extinctions of M at 410 nm, M at 570 nm, N at 410 nm, and N at 570 nm to be 35 800, −63 000, −1000, and −30 500 $M^{-1}\cdot cm^{-1}$, respectively (Váró & Lanyi, 1990b). Figure 1 shows that M decays more rapidly than N; from 2 to 7 s, about half of the N remains, while about 90% of the M disappeared. All the light-induced changes vanished by 62 s (not included in Figure 1). Throughout the time range of the experiments, no increase at 640 nm due to O, whose λ_{max} is at 640 nm, was observed (not shown).

The changes in absorbance between 2 and 7 s and between 7 and 62 s were measured at an interval of 10 nm for 300 to 700 nm and plotted by crosses and triangles, respectively, in Figure 2. The spectrum between 7 and 62 s exhibited a negative peak around 585 nm with a flat part over the 500–450-nm range and no absorption peak around 410 nm. These features resemble those of the N/BR^L visible spectrum reported previously by others (Drachev et al., 1987; Kouyama et al., 1988; Dancshazy et al., 1988). On the other hand, the spectrum between 2 and 7 s showed a positive peak at 410 nm, which is characteristic of M (Lozier et al., 1975; Iwasa et al., 1980).

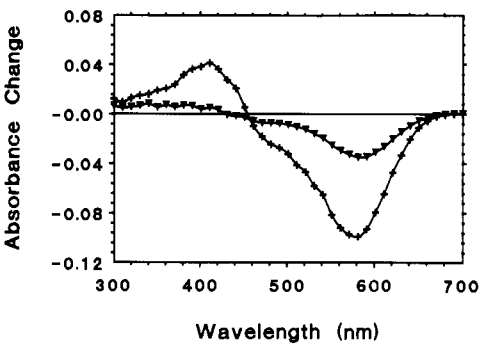

FIGURE 2: Difference absorption spectra in the 300–700-nm region calculated as the absorbance decrease from 2 to 7 s (+) and from 7 to 62 s (▼) after irradiation with >470-nm light for a highly hydrated film of BR^L at pH 10 and 274 K.

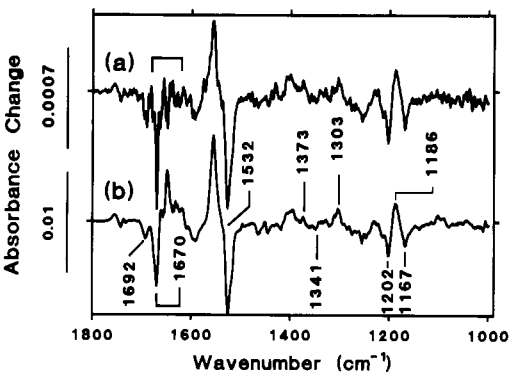

FIGURE 3: FT-IR difference spectra in the 1800–1000 cm^{-1} region recorded for a highly hydrated film of BR^L at pH 10 at 274 K after irradiation with >470-nm light. (a) Spectrum recorded from 7 s. (b) Spectrum recorded from 2 s. The spectra in the 1675–1620 cm^{-1} region (marked by a horizontal line limited by two vertical bars) exceeded the range for the linear response of the absorbance due to ambiguity in the spectral subtraction. The negative bands at 1202 and 1167 cm^{-1} were used to normalize the intensities in Figures 3–5 and 7–9 (see Materials and Methods). The other vibrations are discussed in the text.

FT-IR Spectrum of N. Under identical conditions as described above, the differences FT-IR spectra were recorded from 2 s and from 7 s, respectively, after irradiation. For the measurements, 128 interferograms were summed up over a time of about 1 min after initiation of the recording. The species responsible for these spectra were short-lived under these conditions, because they could not be observed after 1 min, so we assume bR is back in its initial state. Therefore, the cycle of irradiation and recording for 1 min could be repeated 30 times.

The FT-IR spectra in the 1800–1000 cm^{-1} region recorded after 7 s (Figure 3a) and 2 s (Figure 3b) were almost identical except for the 1675–1620 cm^{-1} region where strong absorption due to a large amount of water in the highly hydrated film caused noise. From the visible absorbance data described above (Figures 1 and 2), the spectrum from 7 s (Figure 3a) contained only the N/BR^L spectrum, and the spectrum from 2 s (Figure 3b) was composed mainly of the N/BR^L spectrum with a small (20%) contribution of M/BR^L. Only the 1762 cm^{-1} band in the carboxylic acid region and a broad 1560 cm^{-1} band in the amide II region from M [not shown in figures; see Roepe et al. (1987)] might affect the N/BR^L spectrum.

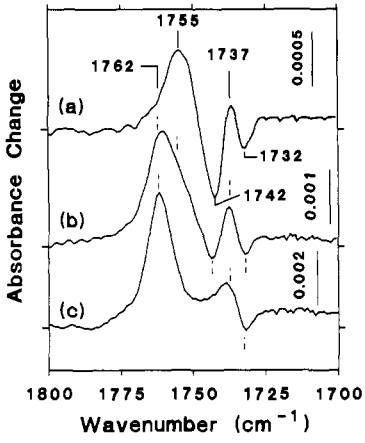

FIGURE 4: FT-IR difference spectra in the 1800–1700 cm^{-1} region of BR^L at pH 10 upon irradiation. (a) Highly hydrated bR (70% water by weight) at 274 K. This spectrum is taken from that in Figure 3b. (b) Humidified bR (50% water by weight) at 274 K. (c) Humidified bR at 230 K. The vertical dashed line represents the same wavenumber as in (a). Vertical solid lines represent the scale bar in absorbance units.

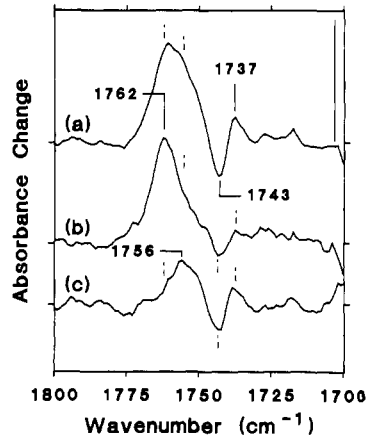

FIGURE 5: N/BR^L difference spectra in the 1800–1700 cm^{-1} region for a humidified bR film at pH 7. (a) 260 K. (b) 230 K. (c) (a) − (b). The vertical dashed line represents the same wavenumber as is labeled. The vertical solid line in the upper right corner represents the scale bar of 0.0015 absorbance unit for (a) and (c), and 0.0009 absorbance unit for (b).

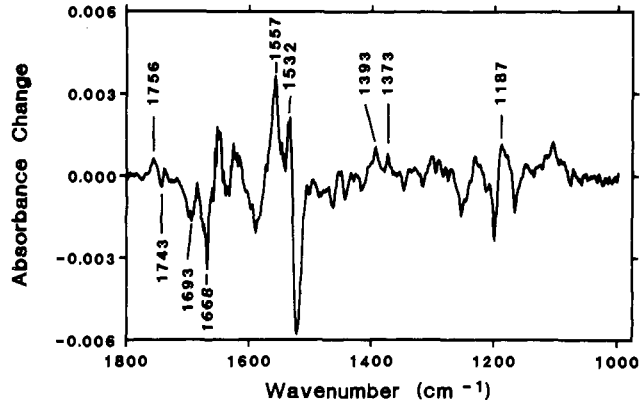

FIGURE 6: N/BR^L difference spectrum at pH 7 in the 1800–1000 cm^{-1} region. The spectrum in Figure 5c was taken from this figure. All the vibrations labeled are discussed in the text.

However, these effects were quite small as discussed below. Since spectrum b of Figure 3 has a better signal-to-noise ratio than spectrum a, we will use the former as the N/BR^L spectrum hereafter.

The N/BR^L spectrum exhibits positive bands at 1373, 1303, and 1186 cm^{-1}, which also appear at similar frequencies in the resonance Raman spectrum of N (Fodor et al., 1988a; Maeda et al., 1986), and are not seen in the resonance Raman spectrum of O (Smith et al., 1983). The ethylenic band at 1532 cm^{-1} observed in the resonance Raman spectrum of N (Fodor et al., 1988a) appears as a shoulder in the N/BR^L spectrum.

Protonated Carboxylic Acid. The C=O stretching vibration of the protonated carboxylic acid appears in the 1800–1700 cm^{-1} region (Engelhard et al., 1985). This region of the N/BR spectrum of a highly hydrated bR (70% water by weight) in Figure 3b is enlarged in Figure 4a. There is a large positive band at 1755 cm^{-1}, a large negative band at 1742 cm^{-1}, a small positive band at 1737 cm^{-1}, and a small negative band at 1732 cm^{-1}.

The spectum of humidified bR with a lower water content (50% water by weight) at pH 10 and 274 K (Figure 4b) has a large positive band at 1762 cm^{-1} due to the presence of M (Siebert et al., 1982), indicating that the low water content in the sample stabilized M. In this preparation, M decayed completely within 10 min (data not shown). Both the relatively small negative band at 1742 cm^{-1} and the positive shoulder around 1755 cm^{-1} are attributed to N. At 230 K (Figure 4c), the humidified bR sample showed neither the negative band at 1742 cm^{-1} nor the positive shoulder at 1755 cm^{-1}, indicating that no N was formed. Thus, the decay of M was too slow for N to be formed during the 8 min for the measurement so that the spectrum of pure M could be obtained. As a result, the positive band at 1762 cm^{-1} appeared to be more symmetric and narrower than in Figure 4b. The positive 1737 cm^{-1} band shifted to a slightly higher frequency (at 1739 cm^{-1}) owing to the removal of the negative band at 1742 cm^{-1}. A negative band at 1732 cm^{-1} was also present. These results demonstrate that M with a positive 1762 cm^{-1} band can be distinguished from N, which is characterized by the positive band at 1755 cm^{-1} and the negative band at 1742 cm^{-1}. As stated above, the N/BR^L spectrum in Figure 4a has a 20% contribution from the M/BR^L spectrum. The 1762 cm^{-1} band of the M/BR spectrum, however, actually appears in the N/BR^L spectrum in Figure 4a only as a small shoulder.

N/BR^L Spectrum at pH 7. At pH 7, we failed to obtain an N/BR^L spectrum having little of the 1762 cm^{-1} band of M for either the highly hydrated bR or the humidified bR. However, the spectrum of M recorded at pH 7 and 260 K for the humidified bR in Figure 5a showed a broader positive band around 1762 cm^{-1} as compared with the corresponding band at 230 K (Figure 5b). Since the negative 1743 cm^{-1} band at 260 K (Figure 5a) is a clear indication of N, the low-frequency side of the 1762 cm^{-1} band is attributed to the 1755 cm^{-1} band of N. Thus, the spectrum measured at 260 K contained a larger proportion of N than that at 230 K in terms of the intensity ratio of those bands characteristic of M and N. The difference between Figure 5a and Figure 5b, which was calculated by subtracting the contribution of M using the 1762 cm^{-1} band to scale the spectrum, should be the N/BR^L spectrum at pH 7 (Figure 5c). The positive band at 1756 cm^{-1} and the negative band at 1743 cm^{-1} were located at frequencies similar to the 1755 and 1742 cm^{-1} bands of the N/BR^L spectrum at pH 10 (Figure 4a). A positive band at 1737 cm^{-1} was observed (Figure 5), but the accompanying negative band at 1732 cm^{-1}, which was observed for M or N at pH 10 (see Figure 4), was quite unclear at pH 7 (Figure 5).

Figure 6 shows the 1800–1000 cm^{-1} region of the N/BR^L spectrum at pH 7 of the spectrum obtained as in Figure 5c. It was quite similar to that at pH 10 (Figure 3). The vibrational bands at 1393, 1373, and 1187 cm^{-1} were located at

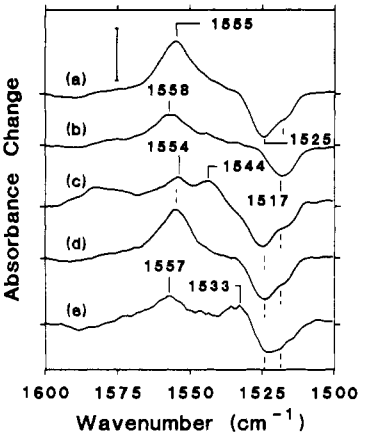

FIGURE 7: N/BR^L difference spectra in the 1600–1500 cm^{-1} region. (a) Native bR at pH 10. (b) C_{15}-Deuteriated bR at pH 10. (c) N-Deuteriated bR at pH 10. (d) ^{15}N-Substituted bR at pH 10. (e) Native bR at pH 7. The vertical dashed line represents the same wavenumber as (a), (b), or (c). The vertical solid line in the upper left corner represents the scale bar of 0.0086, 0.0100, 0.0050, 0.0058, and 0.0026 absorbance units for (a)–(e), respectively.

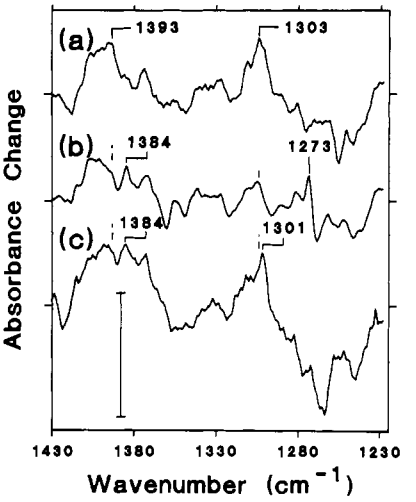

FIGURE 8: N/BR^L difference spectra in the 1430–1230 cm^{-1} region at pH 10. (a) Native bR. (b) C_{15}-Deuteriated bR. (c) N-Deuteriated bR. The vertical dashed line represents the same wavenumber as in (a). The vertical solid line is a scale bar representing 0.0033, 0.0038, and 0.0019 absorbance units for (a), (b), and (c), respectively.

almost the same frequencies (1186 cm^{-1} in Figure 3b; as for the 1393 cm^{-1} band, see Figure 8a). The negative band at 1668 cm^{-1} appeared with a slight shift. The 1532 cm^{-1} band, which was revealed only as a shoulder in N at pH 10 (Figure 3), was clearly observed in the spectrum at pH 7.

Other Characteristic Bands of N. The N/BR^L spectrum at pH 10 exhibited some characteristic bands that were not seen in the spectra of BR^L, K, L, and M. Among them, the band at 1555 cm^{-1} was most pronounced as shown in the 1600–1500 cm^{-1} region spectrum in Figure 7a. C_{15} deuteriation (Figure 7b) depleted most of this, leaving a small band at 1558 cm^{-1} (or two bands at 1561 and 1556 cm^{-1}). N deuteriation (Figure 7c) also depleted a large part of the 1555 cm^{-1} band, leaving two bands at 1554 and 1544 cm^{-1}.

For BR^L, 13-*cis*-bR in dark-adapted bR, and K, the ethylenic bands shift by 5–10 cm^{-1} to lower frequencies upon C_{15} deuteriation but stay at almost the same frequency upon N deuteriation (Braiman & Mathies, 1982; Siebert & Mäntele, 1983; Smith et al., 1987a,b). Resonance Raman experiments on L and N have shown that in the two ethylenic bands only the band at the higher frequency side shifts by 6–8 cm^{-1} upon N deuteriation (Diller et al., 1987; Fodor et al., 1988a).

On the basis of these observations, the C_{15} deuteriation sensitive band that remains at 1544 cm^{-1} after N deuteriation can be assigned to the ethylenic band of the chromophore. The N deuteriation sensitive band that remains at 1558 cm^{-1} upon C_{15} deuteriation may be a protein band. A vibration due to the ϵ-amino group of lysine residues is expected to be present in this region (Krimm & Bandekar, 1986). However, this is not the origin of the 1558 cm^{-1} band we observe because this band is insensitive to ^{15}N substitution (Figure 7d). Instead, it is probably the D_2O-sensitive amide II (Krimm & Bandekar, 1986), although the possibility of a chromophore band cannot be excluded. The 1557 cm^{-1} band of the N/BR^L spectrum at pH 7 (Figure 7e) remained even after C_{15} deuteriation (not shown in figures). It must be the ethylenic band, corresponding to the 1558 cm^{-1} band at pH 10. As stated above, the M/BR^L spectrum exhibits a broad band around 1560 cm^{-1}. However, its molar intensity is about 25% of the 1555 cm^{-1} band of N. Since the molar amount of M was 20% that of N in the FT-IR spectrum of N, it comprises only 5% of the intensity of the 1560 cm^{-1} band of M under the 1555 cm^{-1} band in the N/BR^L spectrum.

On the negative side for BR^L, a 1525 cm^{-1} band was assigned to the ethylenic band of BR^L because it was sensitive to C_{15} deuteriation (Figure 7b) as was noticed previously (Siebert & Mäntele, 1983; Smith et al., 1987a). A shoulder at 1517 cm^{-1} (Figure 7a) remained unchanged upon C_{15} deuteriation (Figure 7b). However, it was not likely to be amide II because it was conserved on N deuteriation (Figure 7c). No candidates for amide II were observed for BR^L in terms of the sensitivity to D_2O substitution.

The spectrum of the amide I region is largely distorted because of the high water content of the sample. However, the 1692 cm^{-1} band on the negative side (Figure 3) was located out of the distorted region and not depleted by either N or C_{15} deuteriation (not shown). It is probably an amide I band and is conserved in the N/BR^L spectrum at pH 7 (Figure 6) at 1693 cm^{-1}. Although another band at 1670 cm^{-1} (Figure 3) [or a 1668 cm^{-1} band at pH 7 (Figure 6)] was located in the distorted region, its intensity far exceeded the noise level and appeared reproducibly. However, about half of its intensity disappeared upon C_{15} deuteriation (not shown in figures). Its assignment to amide I is not straightforward at the present time.

N–H In-Plane Bending Vibration. It has been argued that the frequency of the N–H in-plane bending vibration increases with an increase in the H-bonding strength (Maeda et al., 1985; Baasov et al., 1987; Kitagawa & Maeda, 1989). In the 1430–1230 cm^{-1} region, vibrational bands containing N–H and C_{15}–H in-plane bending modes are present for BR^L (Massig et al., 1982; Siebert & Mäntele, 1983; Smith et al., 1987a; Maeda, Sasaki, Pfefferlé, Shichida, and Yoshizawa, unpublished observations) or for 13-*cis*-bR in dark-adapted bR (Smith et al., 1987b). In these cases, the band which is sensitive to N deuteriation is also sensitive to C_{15} deuteriation. The N–H in-plane vibrational band of L has been shown to be located at 1398 cm^{-1} (Diller et al., 1987). The N–H in-plane bending vibration bands may be picked up as the bands that are sensitive to the deuteriation of the protons at either N or C_{15}. In the N/BR^L spectra, the 1393 and 1303 cm^{-1} bands of native bR (Figure 8a), which are replaced by 1384 and 1273 cm^{-1} bands upon C_{15} deuteriation (Figure 8b), also shift to 1384 and 1301 cm^{-1}, respectively, upon N deuteriation (Figure 8c). Thus, these bands at 1393 and 1303 cm^{-1} can be assigned to vibrations containing N–H and C_{15}–H in-plane bending vibration modes.

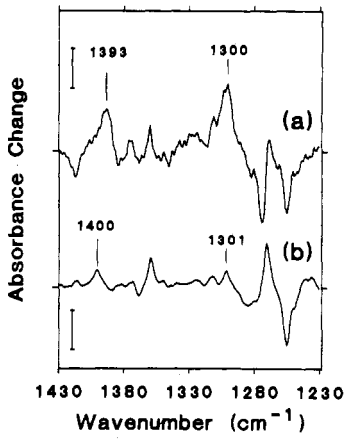

FIGURE 9: Difference spectrum of native bR minus that of C_{15}-deuteriated bR. (a) N/BR^L. (b) L/BR^L. The vertical solid lines at the top and bottom represent the scale bars of 0.00085 and 0.0041 absorbance units for (a) and (b), respectively.

The two hydrogens of C_{12} and C_{15} in 13-*cis*-retinal have a mutual repulsive interaction, which increases the intensities of the bands containing the C_{15}–H in-plane bending mode through a coupling with the C_{12}–H in-plane bending and intervening stretching modes (Curry et al., 1984, 1985). It is interesting to compare the intensities of the C_{15}–H in-plane bending mode between N and L, because these two intermediates give similar in-plane bending bands (Maeda et al., 1986). Since other bands like the C=O stretching vibration are contained in this region (Eisenstein et al., 1987), the intensities of those bands containing the C_{15}–H in-plane bending mode were estimated as the difference between the spectrum of native bR and that of C_{15}-deuteriated bR using the negative 1167 cm^{-1} band as a scale ruler. The results after such subtraction are shown for the N/BR^L (Figure 9a) and L/BR^L (Figure 9b) spectra. The vibrational bands containing the C_{15}–H in-plane bending mode at 1393 and 1300 cm^{-1} for N were more intense than those at 1400 and 1301 cm^{-1} for L.

Discussion

Distinctive Bands of M and N. The N/BR^L spectrum was recorded by taking advantage of a rapid decay of M under highly hydrated conditions at 274 K and of the slow decay of N at pH 10 (Figure 3). The spectrum showed common features with those of N recorded by means of resonance Raman spectroscopy (Maeda et al., 1986; Fodor et al., 1988a; Nakagawa et al., 1990). A small amount of M present together with N did not affect the FT-IR spectrum.

However, a decrease in the water content of the sample to the level which has generally been used for the FT-IR measurements resulted in a large amount of M, which causes difficulties in assessing the spectrum of N (Figure 4b). At pH 10 and 230 K (Figure 4c), where the decay of M was inhibited, the spectrum of M without any contamination of N was obtained. The spectrum of N showed the positive band at 1755 cm^{-1} and the negative band at 1742 cm^{-1} (Figure 4a). These two bands, which were not observed in the spectrum of pure M (Figure 4c), were specific to N. On the other hand, M was specified by the 1762 cm^{-1} band, so that one could identify the presence of N at pH 7 (Figure 5a). Subtraction of two spectra with M and N in different ratios (Figure 5a,b) resulted in the N/BR^L spectrum at pH 7 (Figure 5c), which is almost identical with that at pH 10 (Figure 4a).

Both the 1755 and 1742 cm^{-1} bands in the spectrum of M have been observed by many workers (Dollinger et al., 1986; Roepe et al., 1987; Braiman et al., 1988; Eisenstein et al., 1987). Gerwert et al. (1989) have tentatively proposed that the 1742 cm^{-1} band in the M/BR spectrum belongs to N. In kinetic IR experiments, Siebert et al. (1982) observed two bands at 1755 and 1765 cm^{-1} in the millisecond range, and the 1765 cm^{-1} band decayed more rapidly than the other. This agrees with the present results in view of the fact that N decays later than M. The negative 1732 cm^{-1} band was observed for both M and N at pH 10 (Figure 4c and Figure 4a, respectively). The positive 1737 cm^{-1} band, which was present in the N/BR^L spectrum at both pH 7 and pH 10, was not discerned as a peak in the M/BR^L spectrum at pH 7 (Figure 5b), though present in the M/BR^L spectrum at pH 10 (Figure 4c).

Protonation State of Aspartic Acid Residues. One of the most interesting problems in relation to the function of bR is to know the protonation state of the aspartic acid residues in M and N. According to Braiman et al. (1988), of the four aspartic acid residues buried in the membrane, Asp-96 and Asp-115, which showed negative bands at 1741 and 1735 cm^{-1}, respectively, in the L/BR^L spectrum, were protonated, and Asp-85 and Asp-212, which showed positive bands in the M/BR^L spectrum at 1761 and 1738 cm^{-1}, respectively, were unprotonated in BR^L (the unprotonated state). In our experiments, the positive band at 1737 cm^{-1} appeared independently of the negative band at 1742 cm^{-1} in the M/BR spectrum at pH 10 (Figure 4c). These couples of the positive and negative bands could not be due to shifts of a single residue and should be assigned to different residues.

On the basis of these observations, the negative band of N at 1742 cm^{-1} (Figure 4a) can be assigned to Asp-96. The positive 1762 cm^{-1} band of M (Figure 4b) is also due to Asp-85. The positive 1755 cm^{-1} band of N (Figure 4a) was always accompanied by the negative 1742 cm^{-1} band (1756 and 1743 cm^{-1} at pH 7; Figure 5c). Therefore, this positive band can be assigned to either protonated Asp-96 or Asp-85. In the former case, a protonated Asp-96 in N is hardly compatible with the current idea that Asp-96 is directly involved in proton donation to the Schiff base of M (Holz et al., 1989). More likely, Asp-85 once protonated in M stays in the protonated state while changing its environment, and Asp-96 deprotonates in N. The latter possibility may be verified by finding the carboxylate stretching vibrations by use of [^{13}C]aspartic acid labeled bR for these two aspartic acid residues. A preliminary experiment with labeled bR revealed an isotope-sensitive positive band at 1399 cm^{-1} and a negative band at 1381 cm^{-1}, suggesting Asp-96 is unprotonated and Asp-85 is protonated in N. The negative 1732 cm^{-1} band common to M and N at pH 10 (Figure 4a,c) may be assigned to Asp-115 by analogy with L (Braiman et al., 1988).

In any case, the absence of the 1742 cm^{-1} band in M indicates that Asp-96 neither deprotonates nor undergoes any perturbation in M with the unprotonated Schiff base. Thus, the perturbation of Asp-96 is brought about only through the interaction with the protonated Schiff base. A proton is released during the L to M transition (Drachev et al., 1984; Liu, 1990). Thus, the proton transferred from the Schiff base to Asp-85 at the M stage cannot be the proton to be released; other protons of Arg-82 (Otto et al., 1990), other residues, or water molecules H-bonded with the Schiff base (Hildebrandt & Stockburger, 1984) must be involved in the final proton-releasing step. This proton of Asp-85 remains even in N. Asp-115 underwent changes between BR^L and M and N intermediates at pH 10 but only a little, if any, at pH 7, in view of the behavior of the 1732 cm^{-1} band. Probably, it is not involved in the normal photocycle at pH 7. Our results also confirm the previous observation (Engelhard et al., 1985)

that Asp-96 and Asp-115 in BR^L are protonated even at pH 10.

Peptide Bond of N. The 1555 cm^{-1} band was also characteristic of the N/BR^L spectrum at pH 10 (Figure 7a). It is composed of at least two bands. The one which was located at slightly lower frequencies than 1555 cm^{-1} (Figure 7b) is due to the chromophore, because it is sensitive to C_{15} deuteriation. It may correspond to the 1548 cm^{-1} ethylenic band observed in the resonance Raman spectrum, which shifts to 1542 cm^{-1} upon N deuteriation (Fodor et al., 1988a). A similar 1544 cm^{-1} band in the N/BR^L spectrum was seen in heavy water (Figure 7c). The other was located at 1558 cm^{-1} for C_{15}-deuteriated bR (Figure 7b) and shifted to 1451 cm^{-1} upon N deuteriation (not shown in the figures). Generally, amide II is located in the 1550–1500 cm^{-1} region and shifts to about 1450 cm^{-1} in heavy water (Krimm & Bandekar, 1986). Thus, the 1558 cm^{-1} band can be attributed to amide II. However, no corresponding band of amide II was found in the negative BR^L side in Figure 7. The N/BR^L spectrum at pH 7 (Figure 7e) exhibited only the C_{15} deuteriation sensitive band at 1557 cm^{-1}. This band is probably due to amide II. A similar band has previously been shown to decay later than M in rapid-sweep FTIR difference spectra in the millisecond range (Braiman et al., 1987). In other words, at pH 7 no ethylenic bands were seen in this region. Probably, the ethylenic band at 1532 cm^{-1} gained intensity in place of the band around 1548 cm^{-1}, which was dominant for N at pH 10.

Another characteristic feature of the N/BR spectrum is negative bands at 1692 and 1670 cm^{-1} (Figure 3b). These can be attributed to amide I (the C=O stretching vibration of peptide bond). These bands were noticed previously in the negative side when a probe light vertically polarized to the membrane plane was used in measurements of the M/BR^L spectrum (Earnest et al., 1986; Fahmy et al., 1989). Such a polarization spectrum indicates that some peptide C=O groups point perpendicular to the membrane in BR^L at pH 7. Possibly, the present sample is in a partially oriented state, and particular amide signals appeared by changing the orientation in the transition to N. A similar argument can be applied to the case of the 1558 cm^{-1} band, which does not show any corresponding opposite signal as described above. The vibrational band at 1692 cm^{-1} has been observed for antiparallel β-pleated sheet of poly(L-alanine) (Moore & Krimm, 1976). In any event, these changes associated with the peptide bond are quite local, amounting only to one or two peptide bonds. Alternatively, redistribution of charges in the peptide bond may decrease the infrared intensities of amide I (mainly C=O stretching) and increase those of amide II (mainly N–H bending).

In-Plane Bending Vibrations. The 1393 and 1303 cm^{-1} bands of N (Figure 8a) contain the N–H in-plane bending vibrational mode. These values are similar to those of the corresponding bands of L at 1400 and 1301 cm^{-1} (Diller et al., 1987; Maeda, Sasaki, Pfefferlé, Shichida, and Yoshizawa, unpublished observations). Since H-bonding strength is related to the frequency of the N–H in-plane bending vibration (Maeda et al., 1985; Baasov et al., 1987; Kitagawa & Maeda, 1989), these results mean that the strength of H bonding for the interaction of the Schiff base with the protein of N is almost same as that of L.

The intensities of those bands containing C_{15}–H in-plane bending vibration of L were much less than those for the corresponding bands of N (Figure 9). These may have resulted from the introduction of a twisting of the chromophore in L. It will remove the repulsive interaction between C_{12}–H and C_{15}–H, leading to smaller intensities (Curry et al., 1984, 1985). A twisting of the C_{14}–C_{15} single bond for L has been argued by Fahmy et al. (1989). Such a twisting seems to be relieved in N.

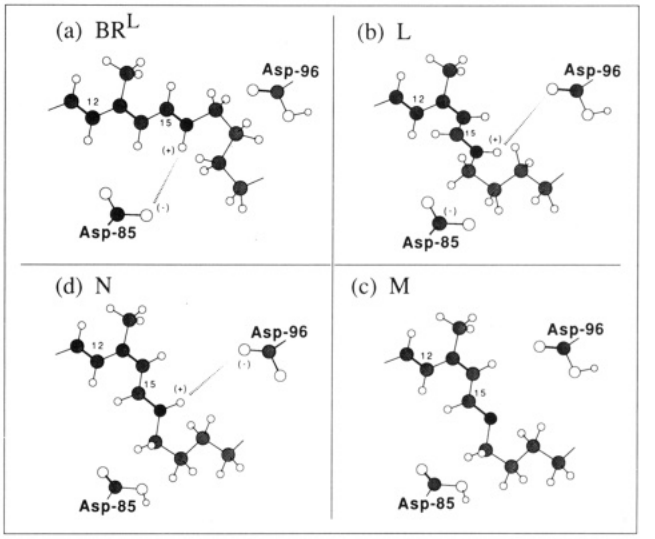

FIGURE 10: Changes of the chromophore binding site in the photocycle of BR^L. Cross-hatched circles, large black circles, large white circles, and small white circles represent carbon, nitrogen, oxygen, and hydrogen, respectively. (a) BR^L. Asp-212 and Arg-82 are omitted. The partner of the H bonding of the Schiff base is depicted as Asp-85 for simplicity, though it could be another residue such as Asp-212, water, or others. (b) L. The 13-cis chromophore has a twisting in the C_{12}–H to C_{15}–H region [see also (c) and (d)]. Asp-96 is tentatively expressed as in its protonated form and directly interacting with the Schiff base. (c) M. The twisting in the chromophore has disappeared, while leaving a small twisting intrinsic for the 13-cis chromophore due to steric hindrance between C_{12}-H and C_{15}-H (Curry et al., 1984). Asp-85 is in its protonated form. (d) N. The chromophore structure is the same as that in (c) except for the Schiff base hydrogen. Asp-96 is in its deprotonated form and depicted as the acceptor for the H-bonding of the Schiff base. Asp-85 is in its protonated form. The dashed line represents H bonding between the Schiff base proton and the oxygen of the aspartic acid residue. (+) and (−) show positive and negative charges, respectively. 12 and 15 are the number of the carbon atom in the retinal.

Chromophore Binding Site in the Photocycle. From the results described above, changes of the chromophore binding site in BR^L, L, M, and N in the photocycle are depicted schematically in Figure 10.

In BR^L (Figure 10a), the N–H bond of the Schiff base points to the side where both Asp-85 and Asp-212 are located (Lin & Mathies, 1989; Henderson et al., 1990). These two negatively charged groups balance with the positive charges of both the Schiff base and Arg-82. The partner for the weak H bonding of the protonated Schiff base (Lugtenburg et al., 1986) may be either of them or another group, such as a water molecule close to the Schiff base (Hildebrandt & Stockburger, 1984).

Upon light absorption, the chromophore isomerizes to the 13-cis form (Braiman & Mathies, 1982), and the N–H bond points roughly to the opposite side in view of C_{14}–C_{15} trans and C=N trans configuration of L (Fodor et al., 1988b; Smith et al., 1984). The positive charge of the Schiff base moves away from the Asp-85 and Asp-212 region and approaches closer to Asp-96. In L (Figure 10b), the chromophore twists in the C_{12}–H to C_{15}–H region by forming strong H bonding of the Schiff base with Asp-96, or another residue intervening between the Schiff base and Asp-96. Fahmy et al. (1989) have argued that the single-bond twists of the chromophore of L are larger than those in BR^L. In other words, the protein residues are orienting so as to give twists in the chromophore

by strong H bonding. Since it is uncertain whether Asp-96 is deprotonated or not in L (Gerwert et al., 1989), the countercharge of the protonated Schiff base is unknown. It is known (Henderson et al., 1990) that Asp-96 is located far from the Schiff base in BR^L, but nothing is known about its location for L.

Schulten and Tavan (1978) have argued that such a twisting decreases the pK_a value of the protonated Schiff base, leading to M (Figure 10c) with an unprotonated Schiff base. The pK_a value of Asp-85 is increased by losing its electrical interaction with the Schiff base by isomerization. The proton from the Schiff base migrates to Asp-85, though present at the opposite side. The deprotonation of the Schiff base then results in the loss of the interaction with the protein, relieving the twisting.

In N (Figure 10d), the Schiff base has its high affinity for the proton restored and accepts the proton from Asp-96, making a strong electrostatic interaction with the deprotonated Asp-96. The chromophore has C_{14}–C_{15} trans, C=N trans configuration as for L (Fodor et al., 1988a). Thus, as the N–H bond still points to the side of Asp-96, it is also depicted as the H-bond acceptor. In this case, the interaction does not cause any twisting, probably owing to the relaxation of the protein moiety. Asp-85 remains to be protonated. Thus, the protonation states of Asp-96 and Asp-85 in N are opposite from those in BR, indicating that those residues are in electrically perturbed states in N. Such a perturbation may be brought into the protein part from the chromophore through the changes in the interaction at the site of the Schiff base. Peptide bonds undergo limited changes in the orientation, environment, or even the charge redistribution. Thus, the conformational change induced in the protein part upon the formation of N is quite localized.

Added in Proof

After submission of this paper, we noticed two related papers. Gerwert et al. (1990) have recorded time-resolved FT-IR spectra of BR^L at 278 K in parallel with changes in the absorbance in the visible region, and observed that the positive band at 1755 cm^{-1}, the negative band at 1741 cm^{-1}, and the positive band at 1186 cm^{-1} belong to the N. These are consistent with our results. Braiman et al. (1991) have recorded time-resolved FT-IR spectra by a stroboscopic technique and found the same changes as above over the time range of N. Their spectrum for N measured at 20 °C was identical with the spectrum at 1 °C presented in this paper. They described by citing their forthcoming paper that the 1755 cm^{-1} band is due to Asp-85. They also described that Asp-96 is deprotonated on the basis of the increased intensity of the 1399 cm^{-1} band for N. According to our observations, this can also be accounted for by the increase in the band intensity containing both N–H and C_{15}–H in-plane bending vibration modes.

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Registry No. Asp, 56-84-8; retinal, 116-31-4.

References

Alshuth, T., & Stockburger, M. (1986) *Photochem. Photobiol. 43*, 55–66.

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

Argade, P. V., Rothschild, K. J., Kawamoto, A. H., Herzfeld, J., & Herify, W. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1643–1646.

Baasov, T., Friedman, N., & Sheves, M. (1987) *Biochemistry 26*, 3210–3217.

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

Braiman, M. S., Ahl, P. L., & Rothschild, K. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5221–5225.

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

Braiman, M. S., Bousché, O., & Rothschild, K. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Curry, B., Palings, I., Broek, A. D., Pardoen, J. A., Mulder, P. P. J., Lugtenburg, J., & Mathies, R. A. (1984) *J. Phys. Chem. 88*, 688–702.

Curry, B., Palings, I., Broek, A., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1985) *Adv. Infrared Raman Spectrosc. 12*, 115–178.

Dancshazy, Zs., Govindjee, R., & Ebrey, T. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6358–6361.

Danon, A., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1234–1238.

Diller, R., & Stockburger, M. (1988) *Biochemistry 27*, 7641–7651.

Diller, R., Stockburger, M., Oesterhelt, D., & Tittor, J. (1987) *FEBS Lett. 217*, 297–304.

Dollinger, G., Eisenstein, L., Lin, S.-L., Nakanishi, K., & Termini, J. (1986) *Biochemistry 25*, 6524–6533.

Drachev, L. A., Kaulen, A. D., & Skulachev, V. P. (1984) *FEBS Lett. 209*, 316–320.

Drachev, L. A., Kaulen, A. D., Skulachev, V. P., & Zorina, V. V. (1987) *FEBS Lett. 226*, 139–144.

Earnest, T. N., Roepe, P., Braiman, M. S., Gillespie, J., & Rothschild, K. J. (1986) *Biochemistry 25*, 7793–7798.

Eisenstein, L., Lin, S.-L., Dollinger, D., Odashima, K., Termini, J., Konno, K., Ding, W.-D., & Nakanishi, K. (1987) *J. Am. Chem. Soc. 109*, 6860–6862.

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

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

Fodor, S. P. A., Ames, J. B., Gebhard, R., van den Berg, E. M. M., Stoeckenius, W., Lugtenburg, J., & Mathies, R. (1988a) *Biochemistry 27*, 7097–7101.

Fodor, S. P. A., Pollard, W. T., Gebhard, R., van den Berg, E. M. M., Lugtenburg, J., & Mathies, R. A. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2156–2160.

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

Gerwert, K., Souvignier, G., & Hess, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9774–9778.

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

Hildebrandt, P., & Stockburger, M. (1984) *Biochemistry 23*, 5539–5548.

Holz, M., Drachev, L. A., Mogi, T., Otto, H., Kaulen, A., Heyn, M. P., Skulachev, V. P., & Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 87* 2167–2171.

Iwasa, T., Tokunaga, F., & Yoshizawa, T. (1980) *Biophys. Struct. Mech. 6*, 253–270.

Kitagawa, T., & Maeda, A. (1989) *Photochem. Photobiol. 50*, 883–894.

Korenstein, R., & Hess, B. (1977) *Nature 270*, 184-186.
Kouyama, T., Nasuda-Kouyama, A., Ikegami, A., Mathew, M., & Stoeckenius, W. (1988) *Biochemistry 27*, 5855-5863.
Krimm, S., & Bandekar, J. (1986) *Adv. Protein Chem. 38*, 183-364.
Li, Q.-Q., Govindjee, R., & Ebrey, T. G. (1984) *Proc Natl. Acad. Sci. U.S.A. 81*, 7079-7082.
Lin, S. W., & Mathies, R. A. (1989) *Biophys. J. 56*, 653-660.
Liu, S. Y. (1990) *Biophys. J. 57*, 943-950.
Lozier, R. H., Bogomolni, R. A., & Stoeckenius, W. (1975) *Biophys. J. 15*, 955-962.
Lugtenburg, J., Muradin-Szweykowska, M., Heeremans, C., Pardoen, J. A., Harbison, G. S., Herzfeld, J., Griffin, R. G., Smith, S. O., & Mathies, R. A. (1986) *J. Am. Chem. Soc. 108*, 3104-3105.
Maeda, A., Ogurusu, T., Yoshizawa, T., & Kitagawa, T. (1985) *Biochemistry 24*, 2517-2521.
Maeda, A., Ogura, T., & Kitagawa, T. (1986) *Biochemistry 25*, 2793-2803.
Massig, G., Stockburger, M., Gaertner, W., Oesterhelt, D., & Towner, P. (1982) *J. Raman Spectrosc. 12*, 287-294.
Mogi, T., Stern, L. J., Marti, T., Chao, B. H., & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4148-4152.
Moore, W. H., & Krimm, S. (1976) *Biopolymers 15*, 2465-2483.
Nakagawa, M., Maeda, A., Ogura, T., & Kitagawa, T. (1990) *J. Mol. Struct. 242*, 221-234.
Oesterhelt, D., & Hess, B. (1973) *Eur. J. Biochem. 37*, 316-326.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667-668.
Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H. G., & Heyn, M. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9228-9232.
Otto, H., Marti, T., Holz, M., Mogi, T., Stern, L. J., Engel, F., Khorana, H. G., & Heyn, M. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1018-1022.
Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987) *Biochemistry 26*, 6696-6707.
Schulten, K., & Tavan, P. (1978) *Nature 272*, 85-86.
Siebert, F., & Mäntele, W. (1983) *Eur. J. Biochem. 130*, 565-573.
Siebert, F., Mäntele, W., & Kreuz, W. (1982) *FEBS Lett. 141*, 82-92.
Smith, S. O., Pardoen, J., Mulder, P. P. J., Curry, B., Lugtenburg, J., & Mathies, R. (1983) *Biochemistry 22*, 6141-6148.
Smith, S. O., Myers, A. B., Pardoen, J. A., Winkel, C., Mulder, P. P. J., Lugtenburg, J., & Mathies, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2055-2059.
Smith, S. O., Braiman, M. S., Myers, A. B., Pardoen, J. A., Courtin, J. M. L., Winkel, C., Lugtenburg, J., & Mathies, R. A. (1987a) *J. Am. Chem. Soc. 109*, 3108-3125.
Smith, S. O., Pardoen, J. A., Lugtenburg, J., & Mathies, R. A. (1987b) *J. Phys. Chem. 91*, 804-819.
Spudich, J. L., & Stoeckenius, W. (1980) *J. Biol. Chem. 255*, 5501-5503.
Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 52*, 587-616.
Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta 505*, 215-278.
Vârô, G., & Lanyi, J. K. (1990a) *Biochemistry 29*, 2241-2250.
Vârô, G., & Lanyi, J. K. (1990b) *Biochemistry 29*, 6858-6865.
Vârô, G., & Lanyi, J. K. (1991) *Biophys. J. 59*, 313-322.

Synthesis of Truncated Amino-Terminal Trimers of Thrombospondin†

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ABSTRACT: Thrombospondin (TSP) is a 450-kDa glycoprotein that is comprised of three identical disulfide-bonded subunits (1152 amino acids) held together near the heparin-binding amino-terminal globular domains. TSP truncated at residue 277 (TSP-277) or 381 (TSP-381) consisted largely of disulfide-bonded trimers when expressed in COS cells or insect cells. In addition, TSP-381 formed heterotrimers with endogenous COS cell TSP. Cleavage of TSP and the truncated mutants in the proteolytically sensitive region between residues 220 and 237 yielded monomeric amino-terminal fragments. Cys-252 and Cys-256 are the only cysteines between residues 238 and 277 and therefore must bridge among subunits. TSP-381 in which Cys-252 and Cys-256 were changed to glycine was secreted efficiently by COS cells but with only a minor portion of the protein in the form of disulfide-bonded trimers. The sequence of TSP between residues 258 and 283 is predicted to form an amphiphatic α-helix. We suggest that assembly of TSP trimers involves formation of an α-helical coiled-coil structure which is stabilized by formation of disulfides.

Thrombospondin (TSP) is a 450-kDa glycoprotein that is found in platelet α-granules (Baenzinger et al., 1971, 1972) and is synthesized and secreted by a variety of cells in culture (Raugi et al., 1982; Mosher et al., 1982; Jaffe et al., 1983; Asch et al., 1986). TSP interacts with a number of macromolecules including collagen type V, fibronectin, histidine-rich glycoprotein, plasminogen, fibrinogen, proteoglycans, heparin, and sulfated glycolipids (Lawler, 1986; Frazier, 1987; Mosher, 1990). TSP has been implicated in platelet aggregation (Leung, 1984; Silverstein, 1986) and also plays a role in the growth (Majack et al., 1986, 1988; Phan et al., 1989) and

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