Complete Identification of C=O Stretching Vibrational Bands of Protonated Aspartic Acid Residues in the Difference Infrared Spectra of M and N Intermediates versus Bacteriorhodopsin†

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ABSTRACT: Fourier transform infrared difference spectra were obtained for the M and N intermediates versus light-adapted bacteriorhodopsin (BR) with site-directed mutant proteins in which aspartic acid residues at positions 96 and 115 were replaced by asparagine. The positive and negative bands at 1740 and 1732 cm^{-1} in the M/BR spectrum are shown to be the superposition of bands due to C=O stretching vibrations of Asp-96 and Asp-115 (a positive band at 1736 cm^{-1} and a negative band at 1742 cm^{-1} of Asp-96, and a positive band at 1742 cm^{-1} and a negative band at 1734 cm^{-1} of Asp-115). The positive band at 1738 cm^{-1} and the negative band at 1734 cm^{-1} in the N/BR spectrum are attributed to Asp-115. On the basis of these results, Asp-115 is protonated in M and N as well as in the ground state. On the other hand, no bands corresponding to Asp-212 were found in the region of protonated carboxylic acid vibration, indicating that Asp-212 remains unprotonated in M and N. The frequencies of the C=O stretching modes of protonated Asp-96 and Asp-115 change in the opposite direction in the BR-to-M conversion relative to the shifts in the BR-to-L conversion, indicating different environmental changes for these residues in L and M.

Bacteriorhodopsin is an intrinsic membrane protein which is composed of seven transmembrane α-helices and functions as a light-driven proton pump in the cellular membrane of *Halobacterium halobium* (Stoeckenius et al., 1979; Henderson et al., 1990). The chromophore of bacteriorhodopsin, retinal, is covalently linked to Lys-216 via a protonated Schiff base. Proton pumping function is carried out by bacteriorhodopsin whose retinal assumes the all-trans configuration (BR).[1] Upon light absorption, the *all-trans*-retinal isomerizes to a 13-cis form followed by stepwise conformational changes of the protein. These changes are distinguished by absorption changes in the visible, and characterized as intermediates called K, L, M, N, and O (Mathies et al., 1991; Lanyi et al., 1993). The L-to-M conversion is coupled to the release of a proton to the extracellular side with accompanying deprotonation of the Schiff base (Liu et al., 1991; Zimányi et al., 1992). In the subsequent M-to-N conversion, the Schiff base is reprotonated, and a proton is taken up from the cytoplasm during the decay of N (Otto et al., 1991).

The involvement of aspartic acid residues in these processes was first pointed out by Fourier transform infrared (FTIR)[1] studies (Engelhard et al., 1985; Eisenstein et al., 1987), since the protonation state of carboxyl groups is monitored by characteristic absorption bands. On the basis of site-directed mutagenesis studies, four aspartic acid residues, Asp-85, Asp-96, Asp-115, and Asp-212, which are buried in the protein, were shown to be of functional importance (Mogi et al., 1988). Further FTIR spectroscopic studies with these mutant proteins revealed protonation changes as well as environmental changes of these Asp residues in the photocycle (Braiman et al., 1988; Gerwert et al., 1989; Maeda et al., 1992). The protonation of the Asp-85 residue upon M formation was shown by assignment of the positive 1762-cm^{-1} band in the M vs BR spectrum (the M/BR spectrum) (Braiman et al., 1988; Fahmy et al., 1992) to this residue. The protonated Asp-85 undergoes an environmental change in the M-to-N conversion, giving a corresponding shift of the band to 1755 cm^{-1} (Pfefferlé et al., 1991; Braiman et al., 1991). The 1742-cm^{-1} band in the negative side of the difference N vs BR spectrum (the N/BR spectrum) was ascribed to the deprotonation of Asp-96 (Bousché et al., 1991; Maeda et al., 1992).

The protonation state of Asp-212 upon M formation was argued on the basis of the assignment of a minor band at 1738 cm^{-1} in the positive side of the M/BR spectrum by the use of mutants of Asp-212 (Braiman et al., 1988; Rothschild et al., 1990). The Asp-212 mutants, however, show different photocycles than wild-type BR (Needleman et al., 1991); hence, the assignment should be regarded as tentative. In addition, opposing evidence was presented on the basis of ^{13}C solid-state NMR data (Metz et al., 1992) and FTIR spectroscopy by use of reconstituted BR in which the carboxylic group of Asp-212 was specifically labeled by ^{13}C (Fahmy et al., 1993).

In the present paper, we aimed to assign minor bands at 1740 and 1732 cm^{-1} in the M/BR spectrum and those at 1738 and 1734 cm^{-1} in the N/BR spectrum by use of the mutant

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[1] Abbreviations: BR, bacteriorhodopsin whose retinal assumes all-trans configuration; FTIR, Fourier transform infrared; SVD, singular value decomposition.

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bacteriorhodopsins D96N, D115N, and D96N/D115N in which either one or both Asp-96 and Asp-115 were replaced by Asn residues. These mutants form M and N intermediates in their photocycles (Sasaki et al., 1992) and show shifts of the minor bands that are helpful in their assignments. In this study, an entire picture for changes of membrane-embedded aspartic acids, throughout the photocycle, becomes clear.

MATERIALS AND METHODS

Sample Manipulation. Wild-type bacteriorhodopsin in the purple membrane was prepared from *Halobacterium halobium* strain ET1001 by the standard method for wild type (Oesterhelt & Stoeckenius, 1973). D96N, D115N, and D96N/D115N bacteriorhodopsins were prepared in the same way from the strain obtained by the recombinant DNA technique applied to *H. halobium* strain L33 (Ni et al., 1990; Needleman et al., 1991; Cao et al., 1991).

The bacteriorhodopsin film for FTIR spectroscopy was prepared by drying purple membrane suspensions in 10 mM borate buffer (pH 10) or in 5 mM phosphate buffer (pH 7), and then highly humidifying by H_2O to a water content of 70% by weight as described by Pfefferlé et al. (1991). The all-trans form of bacteriorhodopsin (BR)[1] was obtained by irradiation of the highly humidified film with >500-nm light for 1 min at 273 K.

Spectral Recording. Spectral recordings were performed in a Nicolet SX60 Fourier transform infrared spectrometer equipped with an Oxford DN1754 cryostat and an Oxford ITC-4 temperature controller. Liquid nitrogen was used as a coolant. The light source for the irradiation was a 1-kW halogen tungsten lamp. The wavelength for the irradiation was selected by Toshiba filters.

Measurement of the M/BR Spectrum. The M intermediate at 230 K was produced by irradiation of BR in the hydrated film at pH 10 with >500-nm light for 1 min. FTIR spectra were measured by recording 128 interferograms before and after irradiation, and the difference was calculated by subtraction between them. The M intermediate was then reconverted to BR by warming to 270 K. These processes were repeated, and the eight difference spectra were averaged.

Measurement of the N/BR Spectrum. The N intermediates of wild-type BR and D115N BR were produced by irradiation of the film at pH 10 with >500-nm light at 270 K. The ground-state spectra were first measured by recording 32 interferograms before irradiation. Forty successive recordings after irradiation, each of which contains five interferograms, followed. Each recording is converted to the difference absorbance spectrum after versus before irradiation. These procedures were repeated 16 times, and the spectra at each time point were averaged. Singular value decomposition (SVD)[1] on the series of these time-dependent spectra was performed, converting these data to a series of wavenumber-dependent spectra (U spectra) and time-dependent spectra (V spectra) (Hug et al., 1990). Among these, two of the U and V spectra exceeded the noise level, indicating two components in the decay kinetics at pH 10 at 270 K. Each of the two V spectra was fitted by two exponential functions with the same two time constants for each spectrum. The spectra corresponding to each of exponential functions were calculated from the U spectra. All these calculations were performed by the SPSERV program (Dr. Csaba Bagyinka, Biological Research Center of the Hungarian Academy of Science, Szeged, Hungary). The spectrum of the slowly decaying component is almost equivalent to the N/BR spectrum whereas that of the fast decaying component mainly contains M (Pfefferlé et al., 1991; see below). This is probably because under these conditions N is approximately in steady-state during the decay of M.

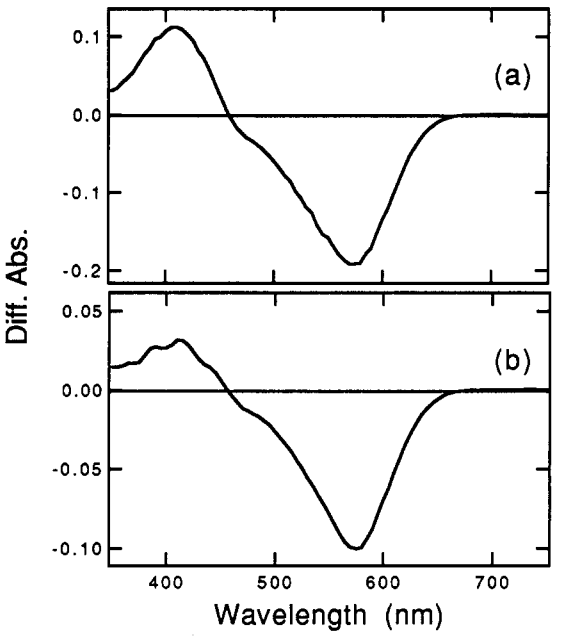

FIGURE 1: Absorption changes in the visible region of D96N after irradiation with >500-nm light at 260 K and pH 7. (a) The fast decaying component whose time constant is 3.6 s. (b) The slowly decaying component whose time constant is 14.8 s.

The N intermediates of D96N and D96N/D115N BR were not produced under these conditions. However, it was possible to produce the N intermediate of D96N by irradiation with >500-nm light at pH 7 at 260 K as described by Sasaki et al. (1992). FTIR spectra were measured in the same way as the wild type as described above. SVD analysis of the data showed two components, and the slower component under these conditions is attributed to the N/BR spectrum (see below). We also measured visible absorption spectra of the corresponding two components under the same conditions. The fast decaying component (Figure 1a) has the strong absorption band around 410 nm that identifies it as M. On the other hand, the slowly decaying component (Figure 1b) exhibits decreased positive absorbance and structure in this region, characteristic of the N/BR spectrum (Fukuda & Kouyama, 1992).

Measurement of the M_N/BR Spectrum. In the photocycle of D96N and D96N/D115N BR at pH 10, a mixture of M and M_N accumulates instead of the N intermediate (Sasaki et al., 1992). The method for the measurements was the same as in the case of the N intermediate of wild-type BR except for the temperature, which was increased to 290 K. SVD analysis was applied to the series of spectra. However, M and M_N were not kinetically separated because both decayed with almost the same rate constant. Therefore, the contribution of M was eliminated by subtracting the M/BR spectra of D96N or D96N/D115N, respectively, as described by Sasaki et al. (1992).

RESULTS

M produced by irradiation of BR of pH 7 at low temperature is always accompanied by contamination of bands due to a small amount of L. Therefore, we produced the pure M by irradiation of BR at pH 10 at 230 K, where L is too unstable to be observed but M is stable during the measurements, as described by Pfefferlé et al. (1991).

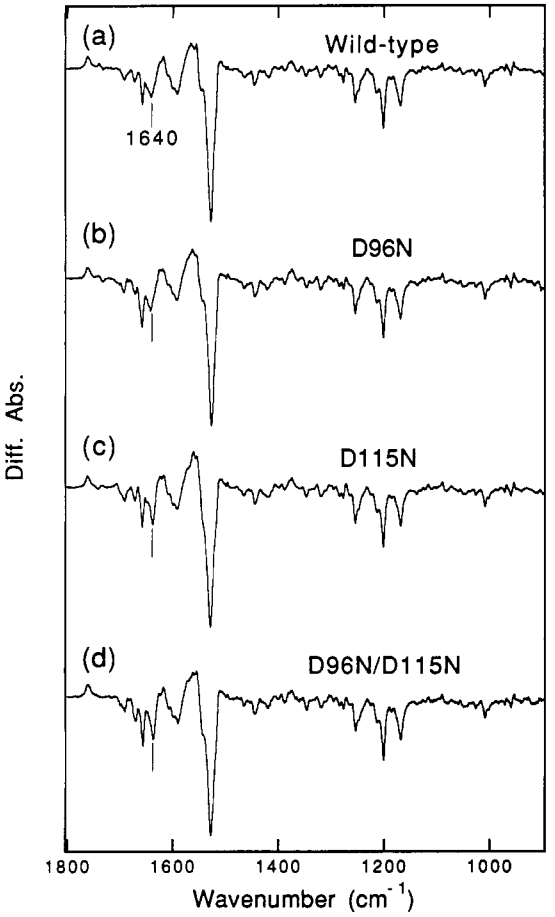

FIGURE 2: FTIR difference spectra of M versus BR for wild type (a), D96N (b), D115N (c), and D96N/D115N (d). They were calculated from spectra measured before and after irradiation of the hydrated film of purple membrane at pH 10 with >500-nm light for 1 min at 230 K. For comparison, the spectra of the mutants were scaled so that all the corresponding bands have the same size as those of wild-type BR. The full scale of the vertical axis is 0.28 absorbance unit for (a).

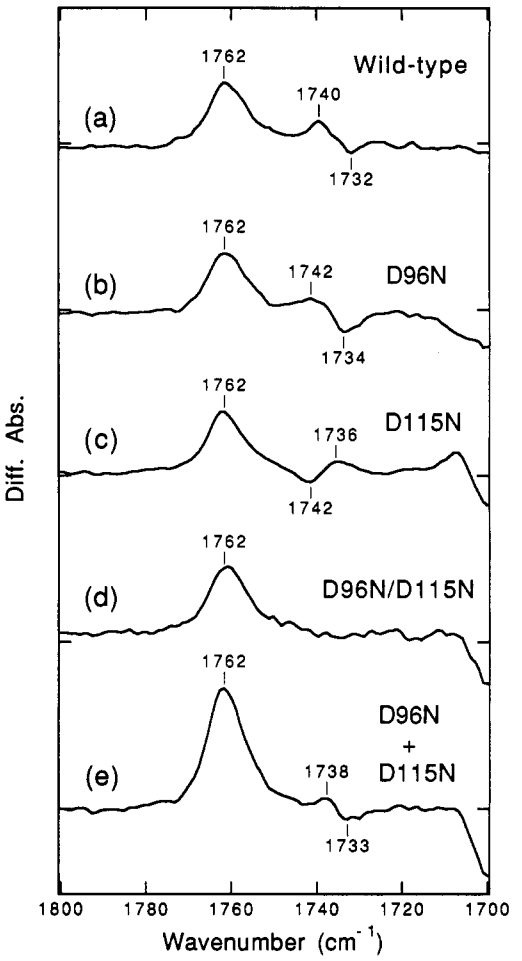

FIGURE 3: Expanded spectra from Figure 2 in the 1800–1700-cm^{-1} region for wild type (a), D96N (b), D115N (c), and D96N/D115N (d). (e) is a spectrum calculated by adding the spectra of D96N (b) and D115N (c). Notice that the intensity of the 1762-cm^{-1} band is twice as large as others. The full scale of the vertical axis is 0.053 absorbance unit for (a).

Difference FTIR spectra were calculated between the spectra recorded before and after irradiation of wild-type, D96N, D115N, and D96N/D115N BR in the hydrated film at pH 10 with >500-nm light for 1 min at 230 K. Figure 2 shows the M/BR spectra of wild type (Figure 2a), D96N (Figure 2b), D115N (Figure 2c), and D96N/D115N (Figure 2d) in the 1800–800-cm^{-1} region. The shapes of all of these spectra are nearly identical. The changes caused by the mutations are observed in the region of the C=O stretching mode of the protonated carboxyl group (COOH vibration) (1800–1700-cm^{-1}) where we expect the effect of the substitutions of asparagine. Since Asp→Asn substitution is expected to cause an about 40-cm^{-1} downshift of C=O stretching vibrational modes (Maeda et al., 1992), the band which disappears in this region upon the substitutions is attributable to the C=O stretching mode of aspartic acid, and the band which appears in the <1720-cm^{-1} region upon the substitution is attributable to the C=O stretching mode of asparagine. Besides these, a negative 1640-cm^{-1} band is more intense in D115N and D96N/D115N than in the wild type and D96N. This band probably belongs to the amide I band which reflects the backbone structure of the protein. Such effects of the mutation of Asp-115 on the backbone structure in L were shown already by Maeda et al. (1992).

The 1800–1700-cm^{-1} region of Figure 2 is expanded in Figure 3. A prominent band at 1762 cm^{-1} has already been assigned to a COOH vibration of protonated Asp-85 (Braiman et al., 1988; Fahmy et al., 1992). In the lower frequency range, there are two small bands at 1740 cm^{-1} on the positive side and at 1732 cm^{-1} on the negative side (Figure 3a). Both bands show small apparent shifts in D96N (Figure 3b), and large changes in D115N, in which the positive and negative bands are shifted to 1736 and 1742 cm^{-1}, respectively (Figure 3c). In the double mutant, D96N/D115N (Figure 3d), these bands have completely disappeared. Therefore, the bands remaining in D96N (Figure 3b) are attributable to Asp-115 while those in D115N (Figure 3c) are attributable to Asp-96. The calculated spectrum obtained by adding Figure 3b and Figure 3c (Figure 3e) exhibits a positive band at 1738 cm^{-1} and a negative band at 1733 cm^{-1}, which almost reproduces the 1740- and 1732-cm^{-1} bands in the M/BR spectrum of the wild type (Figure 3a). The slight decreases in the intensities of the bands in the calculated spectrum could be due to noise. An alternative interpretation for this is minor perturbation by the mutation at position 96 or 115. At least it is possible to say that the bands at 1740 and 1732 cm^{-1} contain only bands attributable to Asp-96 and Asp-115. Since Asp-96 and Asp-115 both have positive and negative bands in the region between 1800 and 1700 cm^{-1}, we conclude that they are protonated in M as well as in BR. The frequency shifts of these residues relative to BR must be caused by environmental changes.

Figure 4 shows the N/BR spectrum of wild type, D96N, D115N, and D96N/D115N. The formation of the N intermediate in the hydrated film of BR was observed at pH

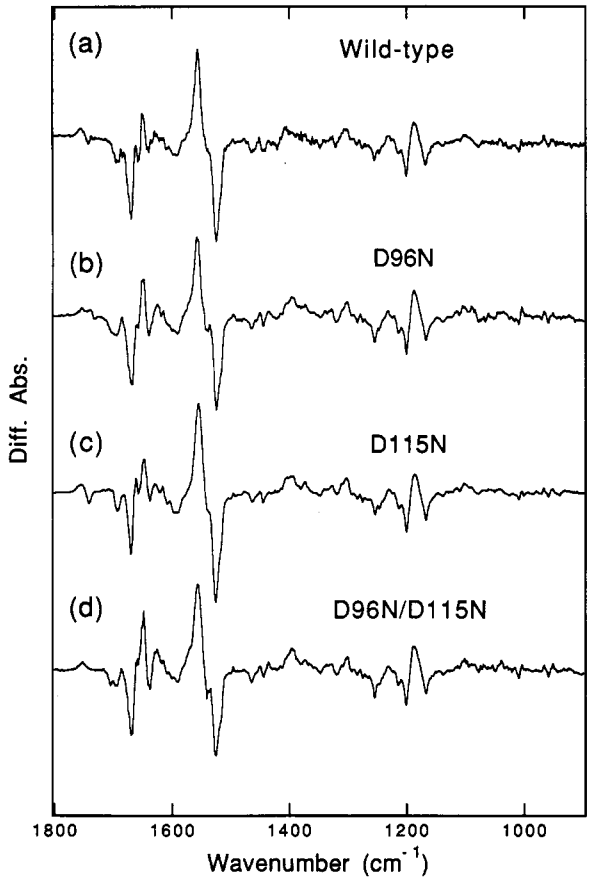

FIGURE 4: FTIR difference spectra of N versus BR for wild type (a), D96N (b), D115N (c), and D96N/D115N (d). They were calculated from spectra measured before and after irradiation of the hydrated film of purple membrane with >500-nm light for 15 s at pH 10, 270 K for wild type (a) and D115N (c) or at pH 7, 260 K for D96N (b) and D96N/D115N (d). Among the two components decaying after the irradiation, the slowly decaying component was attributed to N and presented. For comparison, the spectra of the mutants were scaled so that all the corresponding bands have the same size as those of wild-type BR. The full scale of the vertical axis is 0.113 absorbance unit for (a).

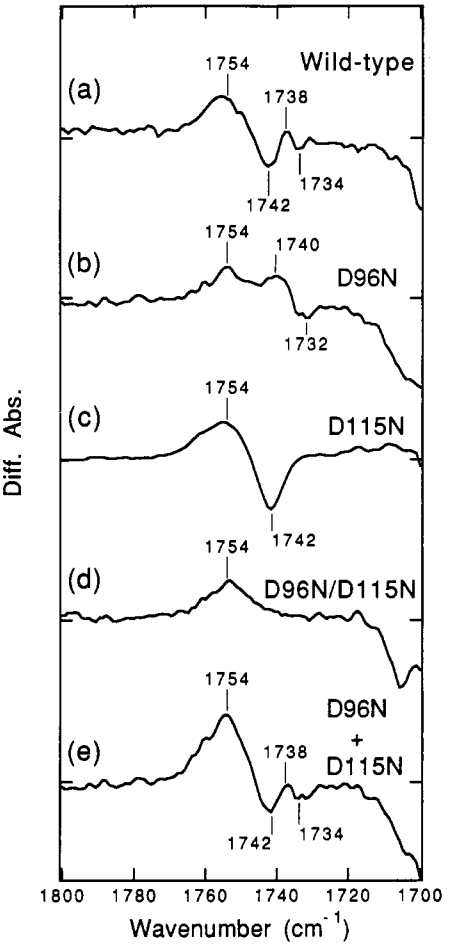

FIGURE 5: Expanded spectra from Figure 4 in the 1800–1700-cm^{-1} region for wild type (a), D96N (b), D115N (c), and D96N/D115N (d). (e) is a spectrum calculated by adding the spectra of D96N (b) and D115N (c). Notice that the intensity of the 1762-cm^{-1} band is twice as large as others. The full scale of the vertical axis is 0.027 absorbance unit for (a).

10 at 274 K by irradiation with >500-nm light (Pfefferlé et al., 1991). The spectrum under these conditions contains a small contribution of M in the fast-decaying component. In the present studies, the N/BR spectrum of wild type (Figure 4a) was obtained by extracting only the slowly decaying component as described under Materials and Methods. By the same methods, the N/BR spectrum of D115N was also obtained (Figure 4c). Under the same conditions, however, D96N does not form N and gives an N-like intermediate, M_N, in which the Schiff base is unprotonated but the protein part takes a structure similar to N. However, despite the absence of Asp-96, the N/BR spectrum in D96N was previously recorded at pH 7 at 260 K (Sasaki et al., 1992). Its spectral shape was almost identical with that of wild-type N, except for the absence of bands of Asp-96. To further confirm that this mutant forms N, we recorded visible absorption spectra under the same conditions. The spectrum of the slowly decaying component corresponds mostly to the N/BR spectrum as shown in Figure 1. We therefore recorded the N/BR spectrum of D96N under these conditions by extracting the spectrum of the slowly decaying component (Figure 4b). The same method was applied to D96N/D115N (Figure 4d). The spectral shapes of these mutants are almost identical to the N/BR spectrum of the wild type, and effects of the mutations are limited to the region of the COOH vibration in the 1800–1700-cm^{-1} region. The small changes in the region around 1650 and 1400 cm^{-1} might be caused by strong absorptions of amide I and the borate buffer, respectively.

Figure 5 shows the expansion of Figure 4 in the 1800–1700-cm^{-1} region. The 1754-cm^{-1} band is a C=O stretching mode of Asp-85 located at a lower frequency than in the M intermediate. This could be due to a change in the environment of protonated Asp-85 in the M-to-N conversion (Pfefferlé et al., 1991; Braiman et al., 1991; Sasaki et al., 1992). The negative 1742-cm^{-1} band, which is attributed to Asp-96, is missing in both Asp-96→Asn mutants D96N and D96N/D115N (Figure 5b,d). The absence of the positive band of Asp-96 in this region indicates that Asp-96, which is protonated in M, deprotonates in the conversion to N. The absence of the negative 1742-cm^{-1} band of the wild type (Figure 5a) in D96N makes the positive band pronounced at 1740 cm^{-1} (Figure 5b), which corresponds to the 1738-cm^{-1} band of wild-type BR (Figure 5a). A positive band at 1738 cm^{-1} and a negative band at 1734 cm^{-1} of wild type (Figure 5a), which remain in D96N, completely disappear in D115N (Figure 5c). In the double mutant D96N/D115N (Figure 5d), these small bands as well as the negative band at 1742 cm^{-1} due to deprotonated Asp-96 are also absent, confirming that these bands are due to Asp-115. The addition of the spectra in Figure 5b and Figure 5c (Figure 5e) reproduces the bands of wild-type BR at 1742, 1738, and 1734 cm^{-1}. Therefore, it is obvious that both the positive 1738-cm^{-1} band and the negative 1734-cm^{-1} band are attributable to the COOH vibration of

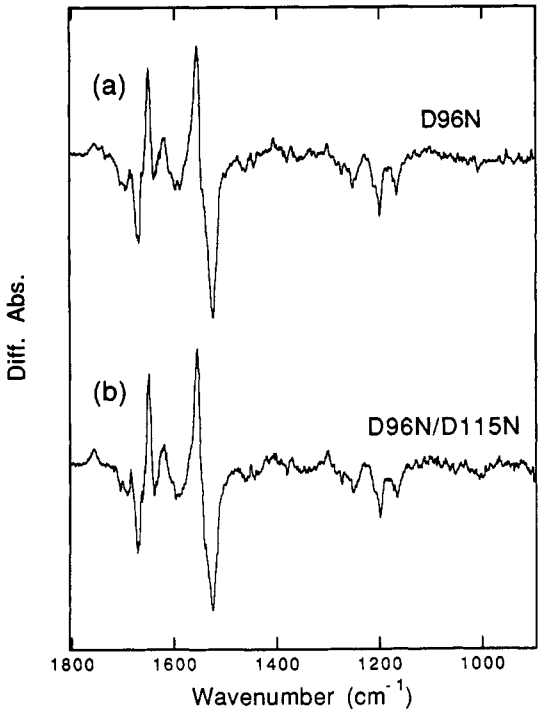

FIGURE 6: FTIR difference spectrum of M_N versus BR for D96N (a) and D96N/D115N (b). The difference spectra were calculated from spectra before and after irradiation of hydrated purple membrane at pH 10 with >500-nm light at 290 K. The spectrum of D96N/D115N was scaled so that all the corresponding bands have the same size as those of D96N for comparison. The full scale of the vertical axis is 0.032 absorbance unit for (a).

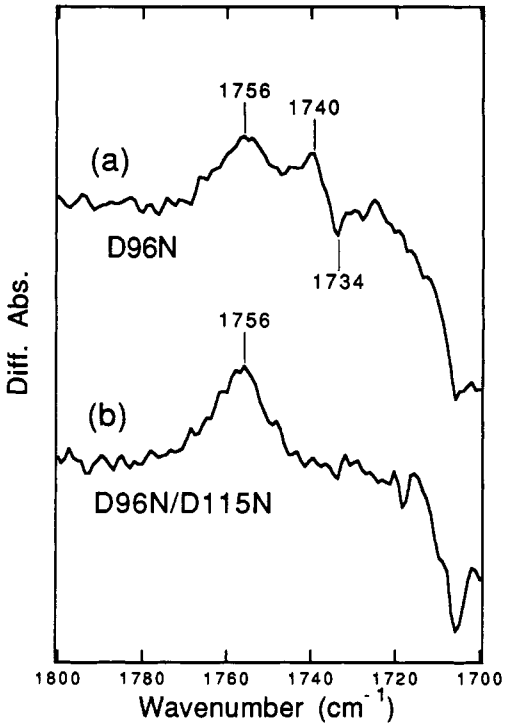

FIGURE 7: Expanded spectra from Figure 6 in the 1800–1700-cm^{-1} region for D96N (a) and D96N/D115N (b). The full scale of the vertical axis is 0.005 absorbance unit for (a).

Asp-115 in N and BR, respectively. The frequency of the bands of Asp-115 in N is almost identical to those in M, indicating that the environment around Asp-115 remains unchanged in the M-to-N conversion.

At pH 10, D96N and D96N/D115N do not produce N in their photocycle. Instead, they show another intermediate, M_N, which has an unprotonated Schiff base like M but a protein structure similar to N (Sasaki et al., 1992). We studied the protein structure of the M_N intermediate of D96N or D96N/D115N at pH 10 to further confirm the assignment in N. The spectral shapes of the M_N/BR spectrum of D96N (Figure 6a) and D96N/D115N (Figure 6b) are almost identical to each other except for the region of the COOH vibration at 1800–1700 cm^{-1}.

This region of Figure 6 is expanded in Figure 7. The M_N/BR spectrum of D96N (Figure 7a) is almost identical to the N/BR spectrum in this region of D96N (Figure 5b). As expected from the N/BR spectra of D96N/D115N (Figure 5d), the M_N/BR spectrum of D96N/D115N (Figure 7b) shows only a single band at 1756 cm^{-1} due to Asp-85, and bands at 1740 and 1734 cm^{-1} in the spectra of D96N (Figure 7a) are missing. This observation confirms that both Asp-85 and Asp-115 experience similar environments in M_N and N.

DISCUSSION

We have intended to produce a complete assignment of the COOH vibration bands of aspartic acid residues in the M/BR and N/BR spectra, by using the D96N, D115N, and D96N/D115N mutant proteins. The gross structural changes in the protein and altered photocycles frequently encountered in mutant studies are very limited for these mutants. BR→M transitions were observed for all of these mutants under the same conditions as for the wild type. The conditions for obtaining the BR→N transition of D115N were the same as for the wild type. Replacement of Asp-96 by asparagine in D96N and D96N/D115N inevitably slows proton uptake because of the absence of Asp-96 as a proton donor of the Schiff base in the M-to-N transition and a proton acceptor in the N-to-O transition (Otto et al., 1989). In D96N and D96N/D115N, proton uptake is coupled to the M_N-to-N transition, because M_N accumulates at pH 10 (Sasaki et al., 1992) rather than N in the wild type. Therefore, to obtain N/BR spectra for D96N and D96N/D115N, the pH had to be lowered to 7 where the M_N-to-N transition was accelerated and revealed the N-to-BR transition. In these cases, proton is taken up directly from the outer milieu, and since reisomerization of the retinal lags behind reprotonation of the Schiff base, N is produced transiently.

The effects of the mutations are restricted almost entirely to the expected COOH vibrations in the 1800–1700-cm^{-1} region. Besides these, only a small conformational change reflected in small deviations in the amide I region appeared in the M/BR spectra of D115N and D96N/D115N. The other parts of the spectra of the mutants are in excellent agreement with that of wild-type BR.

The 1740- and 1732-cm^{-1} bands in the M/BR spectrum of the wild type are ascribed to a superposition of the negative and positive bands of both Asp-96 and Asp-115. The bands of Asp-96 are located at 1736 and 1742 cm^{-1}, and those of Asp-115 are at 1742 and 1734 cm^{-1} on the positive and negative side, respectively. Since Asp-96 and Asp-115 exhibit both negative and positive bands in the region between 1800 and 1700 cm^{-1} in the M/BR spectrum, it is evident that they are protonated in both BR and M. In contrast, no bands except for the 1762-cm^{-1} band which is entirely due to Asp-85 (Fahmy et al., 1992) remain for D96N/D115N, excluding the possibility of the presence of bands due to the protonated Asp-212. It is in the unprotonated state in BR (Marti et al., 1991; Metz et al., 1992); hence, it remains unprotonated in M.

Table 1: Assignment of COOH Vibrations (in cm^{-1}) to Specific Aspartic Acid Residues

	BR	L	M	N
Asp-85	COO^-	COO^-	COOH (1762)	COOH (1756)
Asp-96	COOH (1742)	COOH (1748)[a]	COOH (1736)	COO^-
Asp-115	COOH (1734)	COOH (1729)[a]	COOH (1742)	COOH (1740)
Asp-212	COO^-	COO^-	COO^-	COO^-

[a] From Maeda et al. (1992).

The positive and negative bands at 1738 and 1734 cm^{-1}, respectively, in the N/BR spectrum are both assigned to Asp-115. This shows that Asp-115 is protonated in N. On the other hand, a negative band at 1742 cm^{-1} sensitive to Asp-96 substitution is attributed to the COOH vibration of Asp-96 in BR as described by others (Bousché et al., 1991; Maeda et al., 1991; Sasaki et al., 1992). The absence of the positive band for Asp-96 in this region indicates its deprotonation upon N formation. D96N/D115N shows only the 1754-cm^{-1} band due to Asp-85, and no bands corresponding to Asp-212 were found in the region. Therefore, Asp-212 remains unprotonated in N.

An O/BR spectrum has been presented by the use of a mutant, Y185F (Bousché et al., 1992). The negative band at 1742 cm^{-1} due to Asp-96 is absent, indicating that it is reprotonated in O. The bilobe of the positive band at 1738 cm^{-1} and the negative band at 1734 cm^{-1} remains as in N, indicating the same environment of Asp-115 as in N.

Previously, a C=O stretching vibration at 1738 cm^{-1} in M of wild-type BR was assigned to the protonation of a part of Asp-212 during M formation, on the basis of the lack of this band in Asp-212 mutants (Braiman et al., 1988; Rothschild et al., 1990). The 1738-cm^{-1} band in N and O was also assigned to Asp-212 by use of Y185F (Bousché et al., 1992). However, these mutants exhibit a completely different photocycle. The Asp-212→Asn mutant, especially, does not exhibit an M intermediate which has the unprotonated Schiff base (Needleman et al., 1991; Cao et al., 1993). Two lines of opposing evidence were presented later by ^{13}C NMR (Metz et al., 1992) and FTIR measurements by use of reconstituted BR in which Asp-212 was specifically labeled with ^{13}C (Fahmy et al., 1993). These results suggested that Asp-212 was unprotonated in M and did not contribute to the 1738-cm^{-1} band. Our results support these notions, and further identify the 1740- and 1732-cm^{-1} bands in the M/BR spectrum of wild-type as a superposition of the bands of Asp-96 and Asp-115. Likewise, the 1738- and 1734-cm^{-1} bands in the N/BR spectrum of wild type are identified as originating from Asp-115.

In conclusion, we have assigned all the vibrational bands due to the COOH vibration to the specific aspartic residues in M, N, and M_N. The assignments were accomplished by examining the frequency changes of Asp-96 and Asp-115 upon formation of M and N. Table 1 summarizes the frequencies in the difference bands of the protonated aspartic acid residues 85, 96, and 115 in the BR, L, M, and N intermediates. Upon conversion from BR to L, Asp-96 and Asp-115 are significantly perturbed; Asp-96 shifts upward from 1742 to 1748 cm^{-1}, while Asp-115 shifts downward from 1734 to 1731 cm^{-1}. Followed by the perturbation is M formation as the Schiff base proton is transferred to Asp-85, and a proton is released to the extracellular side. At this stage, Asp-85 exhibits the 1762-cm^{-1} band. In this process, the bands of Asp-96 and Asp-115 shift in the direction opposite to the BR→L conversion; Asp-96 shifts downward to 1736 cm^{-1}, while Asp-115 shifts upward to 1740 cm^{-1}. In the reprotonation of the Schiff base that follows (M→N conversion), the band of Asp-85 exhibits a downshift to 1756 cm^{-1} with the deprotonation of Asp-96, while that of Asp-115 remains at the same frequency.

The cause of the frequency shift of the C=O stretching mode of the carboxyl group can be explained by a change in the through-space distance of the oxygen of the C=O bond to a hydrogen-bonding partner. Its approach results in a lengthening of the C=O bond, which causes the downshift of the stretching mode, as well as a shortening of the C—O bond of the carboxyl group. This is more favorable for the resonant COO^- form. As a consequence, the pK_a of the carboxyl group decreases, and the hydrogen becomes more easily dissociated.[2]

In this manner, the downshifts of the COOH vibrational bands of Asp-96 in the L-to-M conversion and of Asp-85 in the M-to-N conversion might be the consequences of the increased interaction of the C=O bond with hydrogen-bonding partners, leading to the deprotonation of Asp-96 in the M-to-N conversion and of Asp-85 in the conversion to BR, respectively. The downshift of Asp-115 in the formation of L without accompanied deprotonation of the residue can be justified by its transient nature (~1 μs) as it soon undergoes an upshift in M and N. However, the upshift of Asp-96 upon L formation, which is an indication of higher pK_a, is reasonably explained because proton release from Asp-96 must be prevented. Therefore, these environmental changes might be correlated to the switching mechanism of the protein from a state of proton release to another state of proton uptake from the other side, although more clear interpretation of the shifts must await correlation of further experiments with theoretical studies.

[2] Bellamy (1968) discussed the relation of C=O stretching frequency = 1785.5 − 10.5pK_a for various derivatives of carboxylic acids in carbon tetrachloride, in which no hydrogen-bonding is formed. However, H-bonding also lowers the frequency and affects the pK_a values differently.

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