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Structures of Aspartic Acid-96 in the L and N Intermediates of Bacteriorhodopsin: Analysis by Fourier Transform Infrared Spectroscopy[†]

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ABSTRACT: The light-induced difference Fourier transform infrared spectrum between the L or N intermediate minus light-adapted bacteriorhodopsin (BR) was measured in order to examine the protonated states and the changes in the interactions of carboxylic acids of Asp-96 and Asp-115 in these intermediates. Vibrational bands due to the protonated and unprotonated carboxylic acid were identified by isotope shift and band depletion upon substitution of Asp-96 or -115 by asparagine. While the signal due to the deprotonation of Asp-96 was clearly observed in the N intermediate, this residue remained protonated in L. Asp-115 was partially deprotonated in L. The C=O stretching vibration of protonated Asp-96 of L showed almost no shift upon ^2H_2O substitution, in contrast to the corresponding band of Asp-96 or Asp-115 of BR, which shifted by 9–12 cm^{-1} under the same conditions. In the model system of acetic acid in organic solvents, such an absence of the shift of the C=O stretching vibration of the protonated carboxylic acid upon ^2H_2O substitution was seen only when the O–H of acetic acid is hydrogen-bonded. The non-hydrogen-bonded monomer showed the ^2H_2O-dependent shift. Thus, the O–H bond of Asp-96 enters into hydrogen bonding upon conversion of BR to L. Its increased hydrogen bonding in L is consistent with the observed downshift of the O–H stretching vibration of the carboxylic acid of Asp-96.

Bacteriorhodopsin (bR)[1] transports protons across the membrane by utilizing light energy absorbed by the retinylidene chromophore (Stoeckenius et al., 1979). The release of the proton from the membrane occurs at the L–M transition with the deprotonation of the Schiff base (Liu et al., 1990; Váró & Lanyi, 1990b). The deprotonated Schiff base is then reprotonated by Asp-96 in the M–N transition (Holz et al., 1989; Otto et al., 1989; Butt et al., 1989). Proton uptake by the deprotonated Asp-96 from the cytoplasmic side completes the proton transport cycle. The L intermediate is thus a key intermediate, which determines the subsequent reactions for the utilization of light energy to produce a proton gradient.

Maeda et al. (1986) have previously argued that N (previously called L′) is similar to L in the resonance Raman spectrum. Váró and Lanyi (1990a, 1991a) showed that L is in equilibrium with the preceding KL intermediate (Shichida et al., 1983) and also with the next M_1 intermediate (Váró & Lanyi, 1990a). These reversible reactions between the intermediates are due to the small free energy differences among these three intermediates (Váró & Lanyi, 1991c). The

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[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared; BR, light-adapted form of bacteriorhodopsin.

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irreversible step between M_1 and M_2 (Várõ & Lanyi, 1991b) sets a lower free energy for M_2 and N. Thus, in spite of the similarity between L and N, both intermediates may be distinct from each other in chromophore and protein interaction.

Recent Fourier transform infrared (FTIR)[1] studies of bR intermediates (Maeda et al., 1991; Pfefferlé et al., 1991) have indicated that the vibrational bands containing the C_{15}–H in-plane bending mode show lower intensities in L than other protonated 13-cis intermediates like K and N. This fact has been accounted for by the absence of the repulsive interaction between C_{12}–H and C_{15}–H of the 13-*cis*-retinal chromophore by the twisting in the C_{12}–C_{15} region of the retinal. The infrared dichroic ratio for L determined by Fahmy et al. (1989) also shows the twisting of the C_{14}–C_{15} bond. The N–H in-plane bending vibrations in L and N are at higher frequencies than in K and BR (Maeda et al., 1991; Pfefferlé et al., 1991), indicating a stronger hydrogen-bonding interaction with the protein. Thus, the L intermediate is unique in that the twisting of the chromophore appears to align the protonated Schiff base properly to make strong hydrogen bonding with its acceptor.

A difference FTIR spectrum between the L intermediate minus light-adapted bR (BR)[1] has shown (Engelhard et al., 1985) two positive bands at 1749 and 1729 cm^{-1} and a negative band at 1741 cm^{-1}. Engelhard et al. (1985) explained this spectral shape as an overlapped negative band at 1741 cm^{-1} over a wider but less intense positive band which is derived from the transfer of a proton between two carboxylic acids. Eisenstein et al. (1987) then found a carboxylate stretching vibration at about 1400 cm^{-1} by use of bR labeled with [^{13}C]aspartic acid, suggesting the deprotonation of aspartic acids upon L formation. Studies on mutated proteins by Braiman et al. (1988) have revealed, however, that these bands are composed of two bilobic bands. One is from Asp-96, which gives a positive band at 1748 cm^{-1} and the higher frequency side of a negative band at 1741 cm^{-1}. The other is from Asp-115, which is responsible for a negative band around 1735 cm^{-1} and a positive band at 1731 cm^{-1}. These authors have claimed that this pattern arises from the deprotonation of a fraction of Asp-115 and Asp-96 and the simultaneous environmental change of the rest of these aspartic acids. Gerwert et al. (1989) have reproduced the results with D96N. They have explained the results of Asp-96, however, as environmental changes of the carboxyl groups of *all* Asp-96 upon L formation on the basis of the absence of the deprotonation signal of Asp-96 in L. No direct proof by isotope shifts has been given, and structural changes in Asp-96 have not been analyzed well.

Upon conversion to M, which has no proton on the Schiff base, Asp-96 returns completely to the original state (Pfefferlé et al., 1991); the change in Asp-96 reappeared in reprotonated N. These results suggest that the perturbation of Asp-96 in L is exerted from the protonated Schiff base. A recent study (Maeda et al., 1992) has shown that the long-range interaction between the Schiff base and Asp-96 (Henderson et al., 1990) in L involves a small number of water molecules. Proton-transfer studies by use of proteins mutated at Asp-96 (Holz et al., 1989; Otto et al., 1989; Butt et al. 1989; Gerwert et al., 1989) and also time-resolved FTIR studies (Braiman et al., 1991; Bousché et al., 1991) suggested the deprotonation of Asp-96 in N, though crucial proof on the basis of [^{13}C]aspartic acid substitution studies has not been given. Electrostatic interactions between the Schiff base and Asp-96 can thus be raised as a possible cause of a strong interaction in N. Water molecules are also involved in the proton transfer from Asp-96 to the Schiff base (Cao et al., 1991).

To clarify the difference between the interaction in L and N, the structures of Asp-96 in L and N must be examined. The present studies aim at resolving these problems from the isotope shifts and mutational changes of the C=O and O–H stretching vibrational bands of L and N. All of the data strongly suggest that Asp-96 remains protonated and forms increased hydrogen-bonding interaction upon conversion to L, while a small fraction of Asp-115 becomes deprotonated. It is confirmed that Asp-96 is deprotonated in N.

MATERIALS AND METHODS

bR. Bacteriorhodopsin in the purple membrane of *Halobacterium halobium* was prepared by the standard method (Oesterhelt & Stoeckenius, 1974). The clone containing mutated proteins, D96N,[2] D115N, or a double mutant of D96N,D115N, was constructed from strain L-33 by transformation with a shuttle vector containing the modified *bop* gene (Ni et al., 1991; Needleman et al., 1991; Cao et al., 1991).

[^{13}C]Aspartic acid–bR was prepared from cells grown in 200 mL of the synthetic medium containing 1.5 g of DL-[4-^{13}C]aspartic acid (MSD Isotopes, Canada) according to the procedures described by Engelhard et al. (1985) and Dollinger et al. (1986). The ratio of ^{13}C label incorporated into bR, which was determined from the ratio of the peak areas under the negative C=O stretching vibration bands at 1741 cm^{-1} (^{12}C) to 1700 cm^{-1} (^{13}C), was about 50%. Reagents were special grade of Wako (Osaka, Japan). CH_3COO^2H is a product of Aldrich (Milwaukee, WI).

FTIR Spectra. Sample manipulation for FTIR spectroscopy and hydration by ^2H_2O or $H_2{}^{18}O$ were as described previously (Maeda et al., 1992). The L/BR and M/BR spectra were recorded at 170 and 230 K for the humidified samples, respectively, as described by Maeda et al. (1991). The N/BR spectrum was recorded at 274 K for bR at pH 10 under highly hydrated conditions as described by Pfefferlé et al. (1991). The spectra of the different samples or different intermediates were depicted in the figures by adjusting the intensity of the negative band at 1169 cm^{-1} of BR to the same height in the figures (Maeda et al., 1991). The vertically aligned dashed lines in each figure represent the same wavenumber, as labeled. The short horizontal line on the ordinate represents the zero absorbance change. The spectra of acetic acid or propionic acid were measured in a liquid cell with a 1-mm light path length at 294 K.

RESULTS

Effect of ^{13}C Substitution. In Figure 1, the L/BR spectrum in H_2O (a), the L/BR spectrum in ^2H_2O, and the N/BR spectrum in H_2O (c) of [^{13}C]aspartic acid–bR (solid lines) were compared with those of unlabeled bR (dotted lines) in the 1420–1300-cm^{-1} region. For the L/BR spectrum in H_2O (Figure 1a), band intensity around 1400 cm^{-1} decreased upon ^{13}C substitution with a concomitant increase on its lower frequency side around 1389 cm^{-1}. The decrease in intensity at 1400 cm^{-1} was partial as shown in the previous experiments by Eisenstein et al. (1987).

The 1400-cm^{-1} band of the L/BR spectrum is composed of at least two bands. One of them contains the N–H and C_{15}–H

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[2] bR mutants are designated by the wild-type amino acid residue with the standard one-letter code and its position number followed by the substituted amino acid residue. D96N and D115N mean the mutants in which aspartic acid at positions 96 and 115 is replaced by asparagine, respectively.

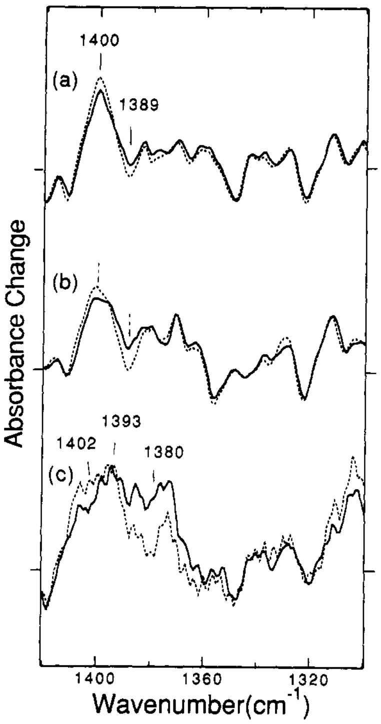

FIGURE 1: Comparison of the spectrum of unlabeled bR (dotted line) with that of [^{13}C]Asp–bR (solid line) between 1420 and 1300 cm^{-1}. (a) L/BR spectrum in H_2O. (b) L/BR spectrum in ^2H_2O. (c) N/BR spectrum in H_2O at pH 10. The whole length of the ordinate represents 0.027, 0.014, 0.030, 0.020, 0.051, and 0.036 absorbance unit for the spectra of the dotted line in (a), the solid line in (a), the dotted line in (b), the solid line in (b), the dotted line in (c), and the solid line in (c), respectively.

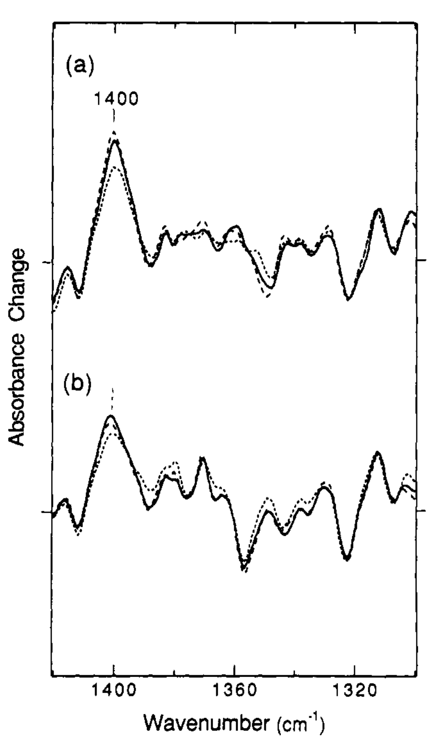

FIGURE 2: Comparison of the L/BR spectrum of wild-type bR (dashed line) with those of D96N (solid line) and D115N (dotted line) between 1420 and 1300 cm^{-1} in H_2O (a) and in ^2H_2O (b). The whole length of the ordinate represents 0.020, 0.032, 0.023, 0.022, 0.031, and 0.025 absorbance unit for the spectra of the dotted line in (a), the solid line in (a), the dashed line in (a), the dotted line in (b), the solid line in (b), and the dashed line in (b), respectively.

in-plane bending vibrational modes (Maeda et al., 1991). Accordingly, it is appropriate to test the intensity decrease in ^2H_2O for the L/BR spectrum (Figure 1b), because the vibration of the carboxylate will remain at the same frequency and the other will disappear. With [^{13}C]aspartic acid–bR in ^2H_2O, an intensity decrease at 1400 cm^{-1}, which was even greater than in H_2O, occurred. However, a large fraction of the intensity still remained around 1400 cm^{-1}, even if one considers the ratio of the ^{13}C labeling to be about half (see Materials and Methods). No other striking effects of ^{13}C substitution were seen in the region between 1420 and 1300 cm^{-1}.

Recently, Pfefferlé et al. (1991) have recorded an N/BR spectrum at pH 10, which shows a negative band at 1742 cm^{-1} and a positive band at 1755 cm^{-1}. The former band was assigned to Asp-96, and the latter was assigned to either Asp-96, if Asp-96 undergoes an environmental change, or Asp-85, if Asp-96 deprotonates in N. The latter possibility requires a ^{13}C-dependent shift around 1400 cm^{-1}. As seen in the N/BR spectrum of Figure 1c, ^{13}C substitution caused a decrease in the intensity of the positive side for N around 1402 cm^{-1}. A large peak remained at 1393 cm^{-1} and is due to a combined mode of C_{15}–H and N–H in-plane bending vibrations (Pfefferlé et al., 1991). Since these L/BR and N/BR spectra were depicted by adjusting the negative BR band at 1169 cm^{-1}, the decrease in intensity at 1402 cm^{-1} of the N/BR spectrum (Figure 1c) is thus much larger than that at 1400 cm^{-1} of the L/BR spectra on the basis of the molar amount (Figure 1a,b). The results show that, upon N formation, Asp-96 deprotonates and Asp-85 remains protonated.

A greater shift for Asp-96 of N than for Asp-115 of L is probably due to the different environments of these two carboxylates. The N/BR spectrum also contains the contribution of the protonation of Asp-85 and -212 (our unpublished results) in the negative side. An apparent greater increase in the intensity around 1380 cm^{-1} than the decrease around 1402 cm^{-1} upon ^{13}C substitution may result from the decrease in the negative intensity due to the protonation of Asp-85 and -212, in addition to the deprotonation of Asp-96. This will also result in the greater shift in the N/BR spectrum.

The replacement of Asp-96 or -115 with asparagine will result in the depletion of the 1400 cm^{-1} band in both H_2O and ^2H_2O, if this band contains the carboxylate stretching mode of either of these aspartic acids. The spectra of D96N (solid line) and D115N (dotted line) were compared with the spectrum of wild-type bR (dashed line) in both H_2O (Figure 2a) and ^2H_2O (Figure 2b). The intensity at 1400 cm^{-1} was almost unchanged in D96N and slightly decreased in D115N. The absence of the contribution of Asp-96 to the 1400-cm^{-1} band can be seen in the previous data [see Figure 3 of Gerwert et al. (1989)]. These results show that the deprotonation occurs only for Asp-115 but not for Asp-96 in L. The possibility that the shift could arise from the changes in tryptophan (Engelhard et al., 1989) is unlikely because the D115N mutation showed a similar decrease.

Effect of Mutations and $H_2{}^{18}O$ Substitution in the 3700–3450-cm^{-1} Region. The L/BR spectrum in the 3700–3450-cm^{-1} region shows a characteristic shape due to the O–H stretching vibrations including that of water (Maeda et al., 1992): a discrete negative band at 3642 cm^{-1}, a broad negative band between 3630 and 3560 cm^{-1}, and a broad positive band between 3560 and 3490 cm^{-1}. A narrow positive band at 3486 cm^{-1}, which is insensitive to ^2H_2O substitution, has tentatively been assigned to the N–H stretching vibration of the peptide bonds. However, the broad band between 3560 and 3490 cm^{-1}, although contributed from the water, is largely composed of a non-water O–H stretching vibrational mode. The effect of

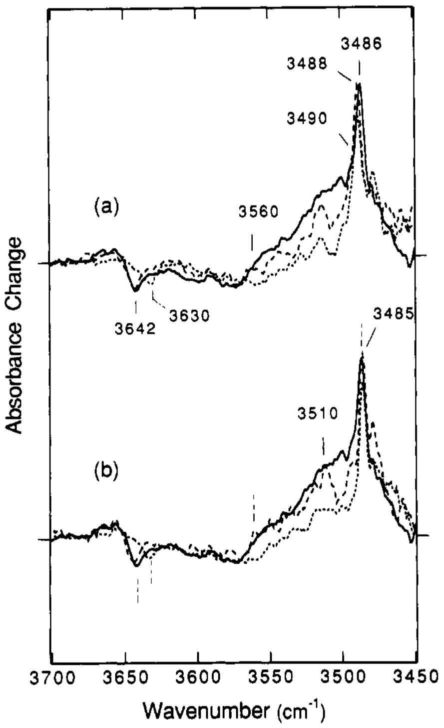

FIGURE 3: Comparison of the L/BR spectrum of wild-type bR in H_2O (solid line) with (a) those of D96N in H_2O (dashed line) and D96N in $H_2{}^{18}O$ (dotted line) and (b) those of D115N in H_2O (dashed line) and D115N in $H_2{}^{18}O$ (dotted line) between 3700 and 2450 cm^{-1}. The whole length of the ordinate represents 0.026, 0.042, 0.031, 0.029, 0.030, and 0.028 absorbance unit for the spectra of the solid line in (a), the dashed line in (a), the dotted line in (a), the solid line in (b), the dashed line in (b), and the dotted line in (b), respectively.

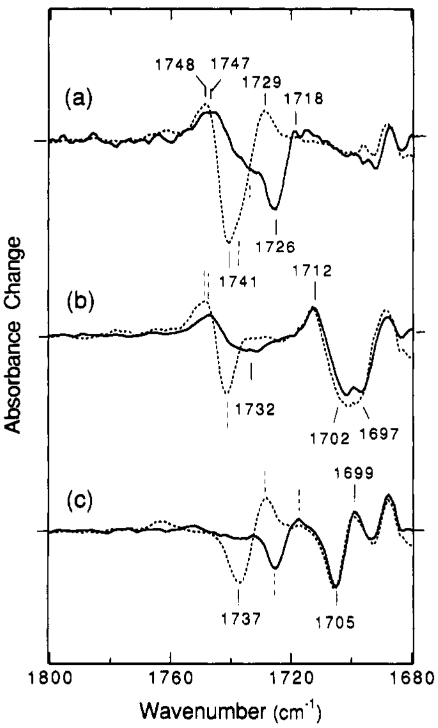

FIGURE 4: Comparison of the L/BR spectrum in H_2O (dotted line) with that in ^2H_2O (solid line) between 1800 and 1680 cm^{-1}. Wild-type bR (a), D115N (b), and D96N (c). The whole length of the ordinate represents 0.020, 0.022, 0.023, 0.025, 0.032, and 0.031 absorbance unit for the spectra of the dotted line in (a), the solid line in (a), the dotted line in (b), the solid line in (b), the dotted line in (c), and the solid line in (c), respectively.

mutating either Asp-96 or Asp-115 on the O–H stretching vibration was tested.

The L/BR spectrum of wild-type bR (Figure 3a; solid line) was compared with that of D96N (dashed line). The negative water band of BR at 3642 cm^{-1} was not affected at all, but the positive band in the 3560–3490-cm^{-1} region and the negative band in the 3630–3560-cm^{-1} region lost their intensities. This means that the O–H stretching vibration band of BR shifts to the lower frequency side upon BR to L conversion. Substitution with $H_2{}^{18}O$ (dotted line) for the D96N sample caused further decreases in the intensity of the wide positive band in the 3560–3490-cm^{-1} region as well as a shift of the negative band at 3642–3630 cm^{-1}, but none of the negative band between 3630 and 3560 cm^{-1}. These $H_2{}^{18}O$-induced changes were the same as those observed for wild-type bR (Maeda et al., 1992). In other words, the intensity decrease in the O–H stretching vibration from the wild-type bR to D96N was not attained by the depletion of the water signal as an indirect result of the structural change in the mutant. The positive narrow band at 3486 cm^{-1} shifted to 3488 cm^{-1} without any loss of the intensity.

In Figure 3b, D115N (dashed line) showed lower intensities in the 3510–3485-cm^{-1} region than native bR (solid line). The decrease in intensity for Asp-115 occurred in the lower frequency region than that for Asp-96. In contrast to D96N, no changes were observed for the positive band between 3560 and 3510 cm^{-1} and for the negative band between 3630 and 3560 cm^{-1}. The 3486-cm^{-1} band shifted to 3485 cm^{-1} in D115N. $H_2{}^{18}O$ substitution (dotted line) depleted the positive wide band in the 3560–3490-cm^{-1} region in the same way as that observed for wild-type bR or D96N.

These results on the mutant proteins indicate that the wide positive band in the 3560–3490-cm^{-1} region is mainly due to water and the protonated carboxylic acids of Asp-96 and -115 and that the wide negative band in the 3630–3560-cm^{-1} region is due to the protonated carboxylic acid of Asp-96. In the double mutant D96N,D115N, the effects of two mutations appear additively (not shown in the figures).

Thus, it is shown clearly that upon L formation Asp-96 remains protonated and makes stronger hydrogen bonding. Asp-115 also shows a protonation signal. Thus, the deprotonation of Asp-115 in L above described is partial, as suggested by the smaller intensity decrease than N upon ^{13}C substitution.

Effect of ^2H_2O Substitution in the 1800–1680-cm^{-1} Region. The negative band in the L/BR spectrum of wild-type bR at 1741 cm^{-1} (dotted line of Figure 4a) is composed of two bands; the one on the higher frequency side is due to Asp-96 and the other on the lower frequency side to Asp-115, in comparison with the spectra for D115N (Figure 4b) and D96N (Figure 4c). These results duplicate those by Braiman et al. (1988) for mutant proteins expressed in *Escherichia coli.*

For wild-type bR, ^2H_2O substitution (solid line) extinguished almost all the intensities of the negative 1741-cm^{-1} band and caused the appearance of a negative band at 1726 cm^{-1} with a shoulder on the higher frequency side. This shoulder was revealed as a negative band at 1732 cm^{-1} of Asp-96 of BR by use of D115N in ^2H_2O (solid line in Figure 4b). The negative 1726-cm^{-1} band in ^2H_2O (solid line in Figure 4a) was assigned to Asp-115 of BR, because it was also observed in the spectrum of D96N (solid line in Figure 4c). The 1732-cm^{-1} band of Asp-96 and the 1726-cm^{-1} band of Asp-115 in ^2H_2O arise by the shifts of the 1741-cm^{-1} band of Asp-96 (dotted line in Figure 4b) and the 1737-cm^{-1} band of Asp-115 (dotted line in Figure 4c) in H_2O, respectively. Thus, the deuteration shifts were 9 cm^{-1} for Asp-96 and 11 cm^{-1} for Asp-115 in BR. The ^2H_2O-induced shifts of the 1762-cm^{-1} band of M and the

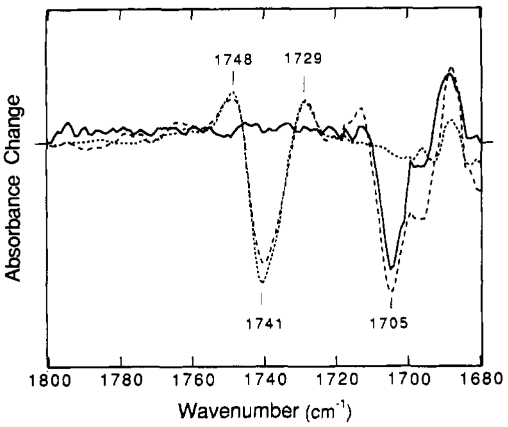

FIGURE 5: Comparison of the L/BR spectrum of the double mutant D96N,D115N (solid line) with those of the summed spectrum of D96N and D115N (dashed line) and wild-type bR (dotted line) between 1800 and 1680 cm^{-1}. The whole length of the ordinate represents 0.008 and 0.006 absorbance unit for the spectra of the dotted line and the solid line, respectively. The spectrum of the dashed line was depicted by summing the two spectra of D96N and D115N after adjustment of the intensities as indicated under Materials and Methods. The same scale represents 0.013 and 0.009 absorbance unit for D96N and D115N, respectively.

1755-cm^{-1} band of N were 12 cm^{-1} (our data; not shown in figures).

On the other hand, the positive band at 1748 cm^{-1} of Asp-96 (Figure 4a) shifted only to 1747 cm^{-1} upon ^2H_2O substitution. The very small deuteration shift was more firmly established with D115N (Figure 4b), in which all the signals are entirely due to Asp-96, as further evidenced by the complete lack of these bands in D96N (Figure 4c). In contrast to a relatively sharp positive band at 1748 cm^{-1} and a negative band at 1741 cm^{-1} of the spectrum in H_2O (5 cm^{-1} in the half-bandwidth for each), the positive band at 1747 cm^{-1} and the negative band at 1732 cm^{-1} (9 cm^{-1} in the half-bandwidth for each) in ^2H_2O are wider; the bandwidth is similar to that of a positive band at 1762 cm^{-1} in the M/BR spectrum (10 cm^{-1}) (our data; not shown in figures). These results suggested that the sharp positive and negative bands at 1748 and 1741 cm^{-1} in H_2O arose from the overlapping of the broad bands. Probably, the real peak of the 1748-cm^{-1} band is present at its lower frequency side and the 1741-cm^{-1} band at its higher frequency side. Thus, the ^2H_2O shift of Asp-96 of BR at 1741 cm^{-1} may be about 12 cm^{-1} as others but that of Asp-96 of L at 1748 cm^{-1} may be less than 1 cm^{-1}, or even may occur to the higher frequency side. A small upshift of Asp-96 of L was seen for BR at pH 10 (not shown).

The positive band of Asp-115 in H_2O is located at 1729 cm^{-1} (dotted lines in Figure 4a,c). However, the corresponding band in ^2H_2O can be defined at 1718 cm^{-1} only as a slightly increased line (solid lines in Figure 4c) above the spectral line before ^2H_2O substitution (dotted line in Figure 4c). Since no distortion appears in the negative band at 1726 cm^{-1}, it is unlikely that the ^2H_2O shift of the positive band of Asp-115 at 1729 cm^{-1} is very small as the case of the positive 1748-cm^{-1} band of Asp-96. The small intensity of the 1718-cm^{-1} band may reflect instead the increased deprotonation of Asp-115 in ^2H_2O. The ^2H_2O shift for Asp-115 in L is thus 11 cm^{-1}.

Additional negative and positive bands were seen at 1705 and 1699 cm^{-1}, respectively, in D96N (Figure 4c). All these bands, though completely insensitive to ^2H_2O substitution, are likely due to frequency changes in Asn-96 as noticed by Gerwert et al. (1989). Use of ^{13}C-D96N bR is necessary to prove it. D115N showed a positive 1712-cm^{-1} band and a wide negative band, which may be composed of two bands at 1702 and 1697 cm^{-1} (Figure 4b). Probably the bilobe at 1712 and 1702 cm^{-1} is due to Asn-115, and the negative band at 1697 cm^{-1}, which shifts slightly upon ^2H_2O substitution, corresponds to the 1658-cm^{-1} amide I band in wild-type bR, which extinguishes its intensity in D115N (not shown).

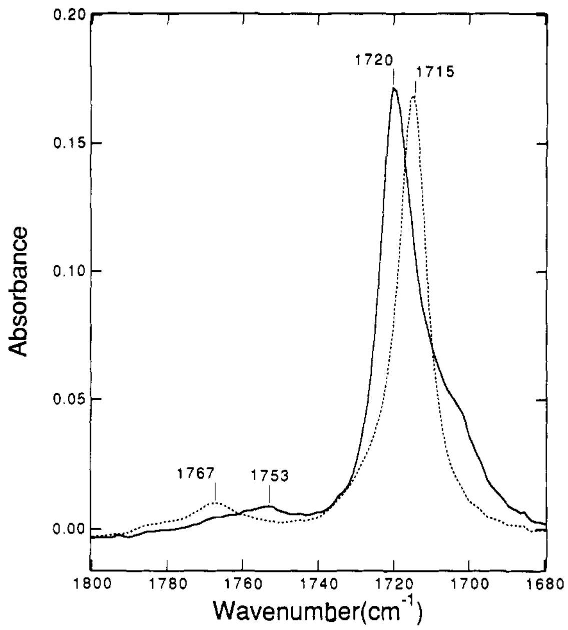

FIGURE 6: Spectra of protonated (dotted line) and deuterated (solid line) acetic acid in carbon tetrachloride. Acetic acid (2 M) in H_2O or ^2H_2O is mixed with carbon tetrachloride. The lower layer (carbon tetrachloride containing acetic acid) was taken for measurement. The spectrum of carbon tetrachloride is subtracted from each.

Summation of these changes in D96N and D115N (dashed line in Figure 5) makes the spectrum almost indistinguishable from that of wild-type bR (dotted line in Figure 5). Thus, the perturbation of Asp-96 and Asp-115 does not affect one another. No negative or positive bands were seen for the double mutant D96N,D115N (solid line in Figure 5) in the 1760–1720-cm^{-1} region, indicating the complete absence of perturbation of any other aspartic acids in L.

Deuteration Effect on C═O Stretching Vibrations of Organic Acid. A shift of the C═O stretching band upon ^2H_2O substitution will originate from the coupling between the C═O stretching and O–H in-plane bending vibrations in the protonated carboxylic acid. It has been well established that organic acids like formic, acetic, and propionic acids form hydrogen-bonded dimers in vapor or an inert solvent like carbon tetrachloride (Nakanishi et al., 1978). The FTIR spectrum of acetic acid in carbon tetrachloride containing H_2O (Figure 6) showed two peaks in the C═O region of the protonated carboxylic acid. The one at 1767 cm^{-1} is due to the monomer, and the other at 1715 cm^{-1} is due to the dimer (Haurie & Novac, 1965a,b; Nakanishi et al., 1978). Upon deuteration, the 1767-cm^{-1} band shifted down to 1753 cm^{-1}, while the 1715-cm^{-1} band shifted up to 1720 cm^{-1}. Thus, only the C═O stretching vibration of the non-hydrogen-bonded monomer shifted down by coupling with the O–H in-plane bending vibration. The upshift of the dimer by deuteration in carbon tetrachloride is consistent with the data presented by Haurie and Novac (1965a,b). A similar result was obtained in the comparison of dry protonated acetic acid vs deuterated acetic acid (not shown). Similar experiments for propionic acid in carbon tetrachloride showed downshifts by 4 cm^{-1} for the dimer and about 9 cm^{-1} for the monomer.

Acetic acid in dioxane showed (Figure 7a) two C═O stretching bands at 1754 and 1728 cm^{-1} (dashed line). In

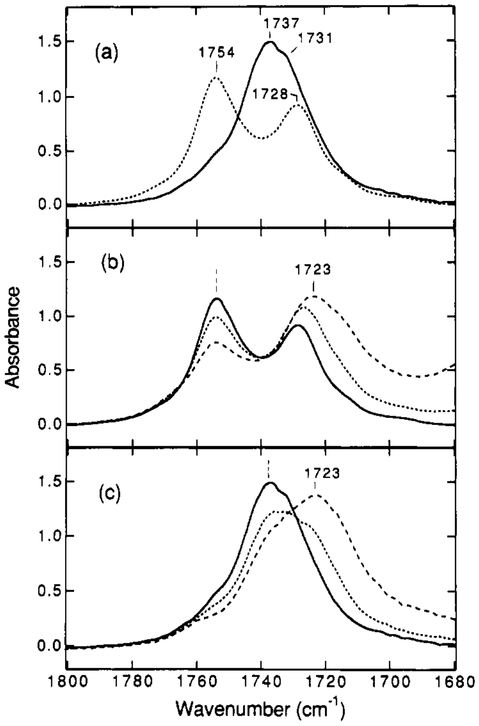

FIGURE 7: (a) Spectra of protonated (dotted line) and deuterated (solid line) acetic acid (2.5%) in dioxane. (b) Changes in the spectrum of CH_3COOH in dioxane upon addition of H_2O. (c) Changes in the spectrum of CH_3COO^2H upon addition of ^2H_2O. In (b) and (c), solid lines are the same as the corresponding spectrum in (a). Dotted lines and dashed lines are the spectra upon addition of 3- and 9-fold molar excess of H_2O or ^2H_2O, respectively.

deuterated acetic acid (CH_3COO^2H) (solid line), these bands merge into a broad single band with a peak at 1737 cm^{-1} and a shoulder at 1731 cm^{-1}, deduced from the second-derivative spectrum. It is likely that the 1728-cm^{-1} band was unshifted upon deuteration, while the 1754-cm^{-1} band shifted to around 1737 cm^{-1}. In analogy with acetic acid in carbon tetrachloride, the higher frequency band may be due to the species which is not hydrogen-bonded and the lower one to that which is hydrogen-bonded. In view of an ability of dioxane to be hydrogen-bonded, the lower frequency band may originate from the interaction of the O–H of the carboxylic acid with the solvent. The higher frequency band indeed decreased in intensity upon addition of H_2O (Figure 7b) or ^2H_2O (Figure 7c), with the concomitant increase of the lower frequency band. Thus, acetic acid which is not hydrogen-bonded showed a large shift of 17 cm^{-1}, and that which is hydrogen-bonded showed little shift.

It is apparent, therefore, that in all the model experiments on carboxylic acids large deuteration downshifts occur for the C═O stretching vibration when the O–H is not hydrogen-bonded but much smaller downshifts, or even slight upshifts, occur when hydrogen-bonded. Upshifts would result from a stronger hydrogen bonding of deuteron than proton.

DISCUSSION

The present studies settle the question of the protonated state of Asp-96 in L. Although the decrease in the intensity at 1400 cm^{-1} observed by Eisenstein et al. (1987) upon L formation was reproduced, it originated from only Asp-115. No signals for the deprotonation of Asp-96 were observed. The presence of the protonated Asp-96 was further shown by the decrease in the intensity of the O–H stretching vibration band of the carboxylic acid in D96N. The uneven bilobic signal of the C═O stretching vibration of the protonated Asp-96 has been explained as the composite of the deprotonation of Asp-96 and the environment change of a small fraction of Asp-96 (Braiman et al., 1988). However, the spectrum of Asp-96 in D115N (Figure 4b) shows both positive and negative bands in H_2O and ^2H_2O with roughly equal intensities. Thus, all lines of evidence for Asp-96 can be merged into the notion that it is protonated in L.

An unusually small deuteration shift (or rather an upshift) for Asp-96 in L was evident, in contrast to the 9–12-cm^{-1} shifts for Asp-115 and Asp-96 in BR, Asp-115 in L, and Asp-85 in M and N. The model carboxylic acid in solution displayed large deuteration downshifts for C═O when the O–H is not hydrogen-bonded, but much smaller downshifts or even slight upshifts when hydrogen-bonded, in contrast to the case of crystalline propionic acid, which shows a normal downshift by 12 cm^{-1} (Umemura, 1977). The anomalous shift for Asp-96 in L can be explained if the O–H bond of Asp-96 participates in hydrogen bonding. Increased hydrogen bonding strength upon formation of L was also deduced from the shift of the O–H stretching vibrations of Asp-96 to the lower frequency side.

The frequency of the C═O stretching vibration is correlated with the hydrogen-bonding strength of C═O···H. Such a comparison should be done for the C═O stretching vibration for deuterated samples, which are virtually free from coupling with the O–H in-plane bending vibration. The shift of the C═O stretching vibration of Asp-96 at 1732 cm^{-1} in BR in ^2H_2O to 1746 cm^{-1} in L could be due to weakened hydrogen bonding. Thus, in Asp-96, hydrogen bonding of C═O···H is weakened but that of O–H···X is strengthened upon BR to L conversion.

The present study is the first direct demonstration of the deprotonation of Asp-96 in N from the isotope shift of C═O stretching of the carboxylate. The previous arguments for the deprotonation of Asp-96 were based on the intensity increase at 1378 cm^{-1} (Gerwert et al., 1990), and also the increase at 1394 cm^{-1} with a concomitant increase of the negative 1720-cm^{-1} band in the case of D96E (Braiman et al., 1991; Bousché et al., 1991) upon increasing the amount of N. However, no appreciable band and ^{13}C shift were observed around 1378 cm^{-1} for N [see Figure 1c and Pfefferlê et al. (1991)]. The increase of the 1394-cm^{-1} band could be the result of an increase in the C═O stretching band as suggested, but a different explanation would be that it is caused by an increase in the intensity of the C_{15}–H in-plane bending vibration at the same frequency (Pfefferlê et al., 1991).

Asp-115 behaved differently from Asp-96. It shows deprotonation in L, in view of the ^{13}C shift of the 1400-cm^{-1} band. However, such a deprotonation occurs only partially, in view of the presence of the positive band at 1729 cm^{-1} due to the protonated Asp-115 and the Asp-115-dependent O–H stretching band. The smaller intensity of the positive band at 1718 cm^{-1} in ^2H_2O than the positive band at 1729 cm^{-1} in H_2O suggests a larger extent of deprotonation of Asp-115 in ^2H_2O. Probably Asp-115 of L has a pK_a value at a nearly neutral pH, and ^2H_2O might cause a subtle pK_a decrease. As support for this, the positive band at 1729 cm^{-1} of Asp-115 of L became smaller at pH 10 (not shown). The two L forms, with deprotonated and protonated Asp-115, are probably in equilibrium under these experimental conditions, but further experiments are required to settle this question. The stronger hydrogen bonding of the C═O···X of the protonated Asp-115, as revealed by the low-frequency shift of the C═O stretching vibration of Asp-115 in L, may be related to its ability to lose the proton by pulling the electron to the carbonyl oxygen. For

Asp-115, no tendency for the increased hydrogen bonding of the O–H bond was observed from the frequency of the O–H stretching vibrations.

The water structural change arising upon L formation was not influenced in D96N as was shown by the remaining water bands: a negative band at 3642 cm^{-1} and a positive broad band between 3560 and 3490 cm^{-1} (Figure 3a). Thus, the substitution of the OH of Asp-96 with NH_2 in D96N will not affect the water structural change upon L formation. No significant changes in the rate for L formation were seen in D96N (Váró & Lanyi, 1991b), supporting this idea.

In transferring the absorbed light energy into the protein, the L intermediate plays a key role in the subsequent deprotonation step of the Schiff base (Schulten & Tavan, 1977; Fahmy et al., 1989; Pfefferlé et al., 1991). A distorted chain conformation close to the Schiff base may be responsible for the decreased affinity of the proton. Recently, stronger hydrogen bonding of a few water molecules in L has been proposed (Maeda et al., 1992). The present study revealed stronger hydrogen bonding of the O–H bond of Asp-96. These interactions disappear completely upon the deprotonation of the Schiff base. Although the relationship of this hydrogen bond to the Schiff base is unknown at present, it must be noted that the L-specific interaction is crucial for establishing strong interaction of the chromophore with the protein.

For D115N, decreased proton pumping and slower proton release have been reported (Mogi et al., 1988; Marinetti et al., 1989). Increased hydrogen bonding of Asp-115 upon L formation or the specific change of the peptide bond in BR of D115N may give another important clue for the characterization of the process of the energy-transfer reaction.

After the submission of the manuscript, Chen and Braiman (1991) presented the L/BR spectrum at room temperature by time-resolved infrared spectroscopy. It is devoid of the positive band at 1748 cm^{-1} of Asp-96 of L. They argued the complete deprotonation of Asp-96 at room temperature, in contrast to low temperature. For establishment of it, the assignment of the carboxylate stretching vibrations by use of [^{13}C]aspartic acid-substituted bR is crucial.

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Registry No. Asp, 56-84-8; CH_3COOH, 64-19-7; ^2H, 7782-39-0.

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