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High-Resolution Solid State ^{13}C NMR of Bacteriorhodopsin: Characterization of [4-^{13}C]Asp Resonances†

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ABSTRACT: Solid state ^{13}C nuclear magnetic resonance measurements of bacteriorhodopsin labeled with [4-^{13}C]Asp show that resonances of single amino acids can be resolved. In order to assign and characterize the resonances of specific Asp residues, three different approaches were used. (1) Determination of the chemical shift anisotropy from side-band intensities provides information about the protonation state of Asp residues. (2) Relaxation studies and T_1 filtering allow one to discriminate between resonances with different mobility. (3) A comparison of the spectra of light- and dark-adapted bacteriorhodopsin provides evidence for resonances from aspartic acid residues in close neighborhood of the chromophore. In agreement with other investigations, four resonances are assigned to internal residues. Two of them are protonated in the ground state up to pH 10 (Asp_{96} and Asp_{115}). All other detected resonances, including Asp_{85} and Asp_{212}, are due to deprotonated aspartic acid. Two lines due to the two internal deprotonated groups change upon dark and light adaptation, whereas the protonated Asp residues are unaffected.

The retinal protein bacteriorhodopsin (BR),[1] found in the purple membrane of *Halobacterium halobium*, acts as a light-driven proton pump [for a review, see Stoeckenius and Bogomolni (1982)]. Recently, a model for the structure of BR has been obtained from high-resolution electron cryomicroscopy (Henderson et al., 1990) showing the atoms of the membrane-embedded part of the protein. The retinal chromophore is bound to Lys_{216} via a protonated Schiff base, and its reversible photoisomerization initiates the BR photocycle. Several intermediates K, L, M, N, and O can be distinguished by their visual absorbance (Lozier et al., 1975; Kouyama et al., 1988). Detailed models for the pumping mechanism of BR, based on different experimental investigations, have been proposed (Engelhard et al., 1985; Braiman et al., 1988; Fodor et al., 1988; Khorana, 1988; Henderson et al., 1990).

The deprotonation of the Schiff base in M is an essential step for the pumping activity (Longstaff & Rando, 1987). The observation of protonation changes of carboxyl groups during the BR photocycle suggested an important role of Asp/Glu residues in proton transport (Siebert et al., 1982; Rothschild et al., 1981). Numerous infrared studies, including the use

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[1] Abbreviations: BR, bacteriorhodopsin; CSA, chemical shift anisotropy; BR_{la}, light-adapted bacteriorhodopsin; BR_{da}, dark-adapted bacteriorhodopsin; CP, cross polarization; NMR, nuclear magnetic resonance; MAS, magic angle spinning; FTIR, Fourier transform infrared.

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of isotopically labeled amino acids of BR, demonstrated the functional importance of Asp carboxyl groups (Engelhard et al., 1985; Eisenstein et al., 1987). The use of BR mutants (Mogi et al., 1988; Marinetti et al., 1989; Gerwert et al., 1989) allowed the assignment of signals to specific amino acids. It was concluded from FTIR difference spectroscopy that Asp_{115} and Asp_{96} are protonated in the initial state. In addition, some evidence was presented that Asp_{212} and Asp_{85} are deprotonated (Braiman et al., 1988). However, in view of the blue-shift of the absorption maximum shown by a mutant in which Asp_{212} is replaced by Asn (Mogi et al., 1988), the protonation state of this group still appears questionable. The group becoming protonated in M was assigned to Asp_{85} (Braiman et al., 1988), and it was suggested that Asp_{96} loses its proton in L (Braiman et al., 1988) or after M (Gerwert et al., 1989).

To obtain further information on the Asp residues in the initial state, we recently studied BR containing [4-^{13}C]Asp by solid state NMR spectroscopy (Engelhard et al., 1989), complementary to the infrared investigations. Signals of single amino acids could be resolved and partly assigned to specific Asp residues by the use of BR mutants (Engelhard et al., 1990). In contrast to FTIR difference spectroscopy, which can only detect spectral changes during the photocycle, absolute spectra are obtained. In principle, this method should allow the investigation of the carboxyl groups in different BR states.

The powerful possibilities of solid state CP-MAS NMR in the investigation of membrane proteins such as BR were reviewed by Smith and Griffin (1988). For example, detailed information about the conformation, the chemical environment, and the isomerization state of the chromophore was gained by reconstituting BR with ^{13}C-labeled retinals or by incorporating biosynthetically ^{15}N-labeled lysines (Harbison et al., 1984, 1985, 1988; De Groot et al., 1988; Smith et al., 1989a). In particular, the chemical shift anisotropy of assigned groups can be interpreted on the molecular level (De Groot et al., 1989, 1990). In our earlier ^{13}C NMR studies, we used the isotropic chemical shift of the different resonances to assign the protonation state of Asp residues (Engelhard et al., 1989). Although lines of protonated groups are expected to be located at higher fields, as compared to an aspartate, an additional discrimination is still needed. The chemical shift anisotropy provides further information about their protonation state. From several studies on small compounds, it is well established that protonation of a carboxyl group results in a significant upfield shift of σ_{22} (Ackerman et al., 1974; Pines et al., 1974; Griffin et al., 1975; Chang et al., 1974). In this paper, by evaluating the side-band intensities, we determine the CSA of the Asp resonances, providing a means to assign the protonation state of Asp residues in BR.

It is thought that at least one deprotonated Asp in BR plays a role in forming the counterion of the Schiff base (Subramaniam et al., 1990; Otto et al., 1990). Recently, on the basis of extensive NMR studies of BR and model compounds, a complex counterion has been proposed (De Groot et al., 1989), which might involve Asp_{85}, Asp_{212}, and Arg_{82}. The interaction of the Schiff base with nearby deprotonated Asp residues can be expected to differ from *all-trans*- to 13-*cis*-retinal and might therefore influence the ^{13}C resonance of the corresponding Asp. Since the retinal isomer content varies between dark-adapted and light-adapted BR (BR_{da} and BR_{la}) (Scherrer et al., 1989; Soppa & Oesterhelt, 1989), it should be possible to identify carboxyl group(s) near the Schiff base.

The overlap of Asp resonances within a small range of σ_{iso} and the contribution of the naturally abundant ^{13}C signals of the peptide backbone carbons can partly be resolved by making use of the different relaxation behavior. It is expected that internal Asp residues are in a more rigid environment. This should lead to a larger T_1 relaxation time and to a better cross-polarization efficiency compared to more mobile groups. By an appropriate choice of the delay time in inversion–recovery spectra, we could suppress the broad band of naturally abundant ^{13}C underlying the Asp signals. The comparison of spectra obtained with and without CP (using in both cases the same short recycle delay) provides additional information on mobility and is used to further characterize the resonances.

Materials and Methods

Purple membrane with wild-type BR was isolated from *Halobacterium* sp. GRB (Soppa & Oesterhelt, 1989) and *H. halobium* (strain R_1M_1). [4-^{13}C]Asp was incorporated by growing the bacteria in a synthetic medium as described earlier (Engelhard et al., 1985, 1990). The biosynthetic pathway in halobacteria also results in a large enrichment of the ^{13}C label at the C-11 position in Trp (Engelhard et al., 1989). The dipeptide model compounds (Ala–Ala, Asp–Ala, Ala–Asp) were obtained from Sigma GmbH, Deisenhofen, Germany.

All spectra were taken on a Bruker CXP300 spectrometer (Bruker GmbH, Karlsruhe) operating at 75 MHz for ^{13}C. Standard cross-polarization (CP), magic angle spinning (MAS) techniques were applied with a 1.5-ms mixing time, 3.5-μs 90° pulse time, 50-ms acquisition time, and a decoupling field equivalent to 75 kHz. About 50 mg of lyophilized sample was filled into the rotor for each experiment. An equal amount (w/w) of 0.1 M borate buffer of appropriate pH was added. Spectra of [4-^{13}C]Asp-enriched and native BR were recorded with different spinning frequencies (1900, 2780, and 3250 Hz). For each frequency, side-band intensities were determined from the difference between the two spectra, and the CSA tensor elements were evaluated according to the method of Herzfeld and Berger (1980). Measurements were performed at 40 and −5 °C and at room temperature at a pH of 7 and 10.

For the comparison of BR_{da} with BR_{la}, about 30 mg of purple membrane was transferred into a transparent sapphire rotor. The rotor was kept in the dark for 24 h at 20 °C, and subsequently the CP-MAS spectrum was recorded with a total acquisition time of 12 h, keeping the sample temperature at −10 °C. The sample was subsequently illuminated for 3 min from all sides outside the magnet with a slide projector. The sample can be light-adapted to a very high extent, because spinning the rotor at 3.25 kHz leads to a thin film of hydrated purple membrane at the wall of the rotor. The spectrum of the light-adapted sample was then taken under identical conditions.

For the T_1 relaxation studies, an inversion–recovery pulse sequence (CP–90°$_x$–τ–90°$_x$) was used. This leads to signals going from negative to positive intensities as the delay time increases. The time at which the signals equal zero can be used for an estimation of T_1. A quantitative determination of the time constant could not be undertaken because of the length of time necessary to obtain spectra at long delay times. In addition, at long delay times ($\tau \gg T_1$) the intensities are no longer CP enhanced.

We also used a modified pulse sequence (Torchia, 1978), which has been already applied in the case of BR (Harbison et al., 1985). It combines CP with the inversion–recovery method. The magnetization which was produced by CP decays to zero by an appropriate phase cycling sequence [for details, see Torchia (1978)]. This would, in principle, allow the evaluation of T_1 without the necessity of going to long delay times. However, the quantitative determination of the time

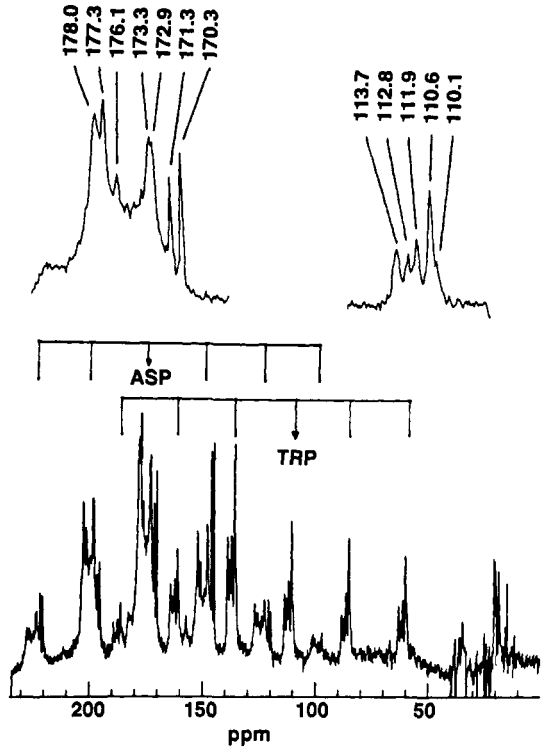

FIGURE 1: ^{13}C CP-MAS difference spectrum between [4-^{13}C]Asp/[11-^{13}C]Trp-labeled and unlabeled BR at 1900 Hz spinning frequency, pH 7, and room temperature. The spectrum shows the center bands (denoted →ASP and →TRP) together with series of side bands as well as the enlarged center band regions of the Asp and Trp signals.

constant is hindered by the overlap of resonances, and we found that no important additional information could be gained. However, the qualitative relaxation differences obtained by the simple inversion–recovery method could be confirmed.

RESULTS

Chemical Shift Anisotropy. Figure 1 shows the difference between the ^{13}C CP-MAS NMR spectra of [4-^{13}C]Asp-labeled and unlabeled dark-adapted BR at 1900 Hz spinning speed. The subtraction constant was choosen to minimize the difference in the aliphatic region. The remaining peaks are due to some label in aliphatic side chains (Engelhard et al., 1989) and the fact that one subtracts large signals which are very sensitive to experimental conditions (e.g., water content and spectrometer adjustment). As previously reported (Engelhard et al., 1989), growing the bacteria in a medium containing [4-^{13}C]Asp also leads to a ^{13}C label at the 11-position of Trp. The resonances of these groups are located between 110 and 114 ppm, and the carboxyl resonances are found between 170 and 178 ppm.

Two series of side bands are clearly discernable. The CSA parameters can be extracted from the ratio of the intensities of the side bands and the center band. However, the overlap of different resonances makes it difficult to accurately determine these ratios. Errors are estimated by taking the intensities at extreme limits, i.e., from the zero baseline or from the minima between the lines, and calculating the effect on the σ_{ii}. This leads to an uncertainty for the tensor smaller than ±10 ppm. The differences we discuss, however, are larger than 20 ppm and are, therefore, beyond these errors.

Figure 2 shows the CSA patterns of the Asp resonances. The mean values of the CSA tensor elements of wild-type BR are summarized in Table I. The data are based on the evaluation of spectra at three different spinning speeds (1900, 2870, and 3250 Hz). The width of the CSA ($\sigma_{11}-\sigma_{33}$) is found

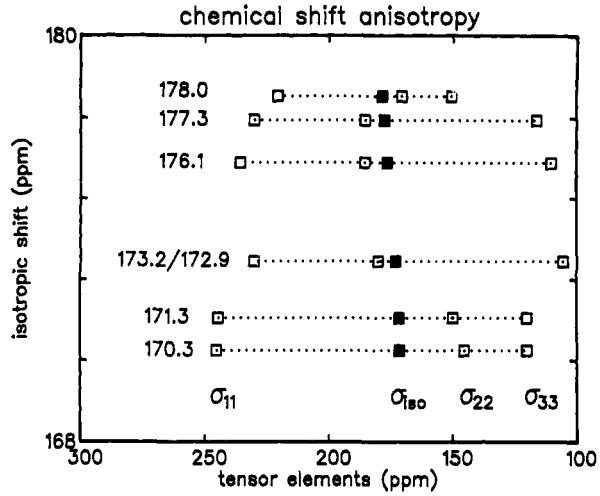

FIGURE 2: CSA principal tensor elements for the [4-^{13}C]Asp resonance as determined by analyses of the side bands. The values are identical for pH 7 and 10 as well as for room temperature and -5 and 40 °C. The errors in the tensor elements, caused by the uncertainty of peak intensity determination, are in the order of <10 ppm.

Table I: Principal Elements of the Chemical Shift Tensor for the [4-^{13}C]Asp and [11-^{13}C]Trp Resonances of Wild-Type BR[a]

residue	σ_{iso}	σ_{11}	σ_{22}	σ_{33}
C-terminal Asp_{242}	178.5	220	170	150
external Asp	177.0	230	185	115
Asp_{212}	176.0	235	185	110
Asp_{85} (*all-trans*-retinal)	173.3	230	180	105
Asp_{85} (13-*cis*-retinal)	172.9	230	180	105
Asp_{96}	171.3	245	150	120
Asp_{115}	170.3	245	145	120
Trp	114.0	195	130	20
Trp	113.0	180	130	30
Trp	112.0	190	130	15
Trp	110.5	190	120	15

[a] The errors in the tensor elements, caused by the uncertainty of peak intensity determination, are on the order of <10 ppm. The chemical shift anisotropies were found to be identical for pH 7 and 10 as well as for room temperature and -5 and 40 °C.

to be approximately 125 ppm for all Asp resonances except for the signal at 178.5 ppm, which has a significantly smaller width (70 ppm). A somewhat reduced CSA width is also found for the resonance at 177.5 ppm (115 ppm). Two groups of CSA patterns reflecting different relative positions of the principal elements are found (Table I). For the signals at 170.3, 171.3, and 178.5 ppm, the difference ($\sigma_{22}-\sigma_{33}$) is smaller than ($\sigma_{11}-\sigma_{22}$), whereas for the other resonances the reverse relation holds. Changing the temperature from 23 to 40 and −5 °C does not alter the CSA patterns. Also, at pH 10, no difference was found for the CSA of any of the Asp resonances.

The principal tensor elements of [11-^{13}C]Trp differ somewhat for the individual resonances (Table I): the resonances at 113.7, 111.9, and 110.6 ppm show a larger width ($\sigma_{11}-\sigma_{33}$ = 175 ppm) than the resonance at 112.8 ppm ($\sigma_{11}-\sigma_{33}$ = 150 ppm). Again, the experimental conditions (pH 7, pH 10, −5 °C, +40 °C) had no influence on the CSA of the Trp signals. We also recorded spectra of dipeptide model compounds of which the carboxyl shift anisotropies are summarized in Table II. The CSA patterns are typical for amino acid carboxyl groups (Veeman, 1984). The sensitivity of σ_{22} with respect to protonation is clearly documented in an upfield shift larger than 20 ppm.

Light Adaption. Figure 3 shows the difference spectra between labeled and native BR in the light- and dark-adapted state, together with the difference of these two spectra. The

Table II: Principal Elements of the Chemical Shift Tensor of Model Compounds Containing Aspartic Acid[a]

model compound	σ_{iso}	σ_{11}	σ_{22}	σ_{33}
Ala–Ala				
C-terminal COO^-	180	237	194	108
Ala–Asp				
C-terminal COO^-	178	237	186	112
side-chain COOH	174	251	158	113
Asp–Ala				
C-terminal COO^-	178	243	176	115
side-chain COOH	175	244	166	114

[a] The CSA was evaluated from spectra at different spinning speeds (data not shown).

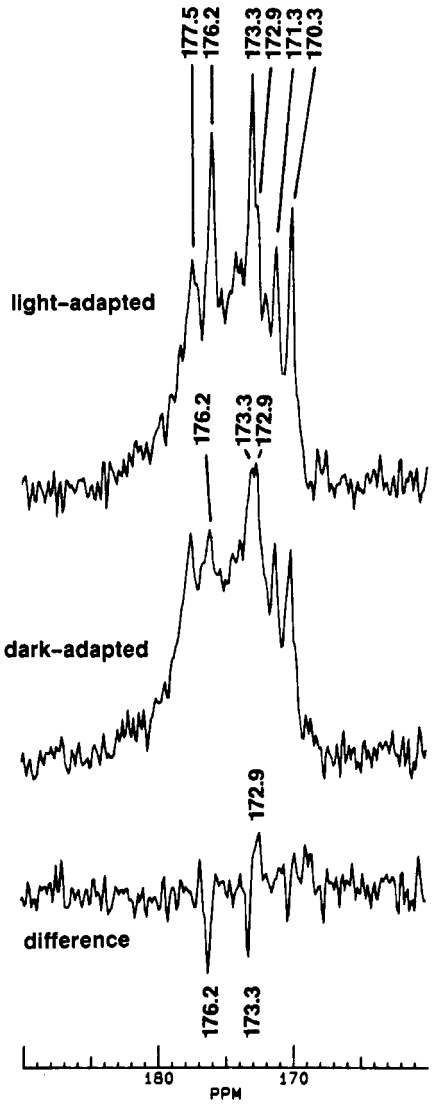

FIGURE 3: ^{13}C CP-MAS difference spectra between [4-^{13}C]Asp-labeled and unlabeled BR in the light-adapted and dark-adapted state, together with the difference between these spectra, showing the effect of light adaption on the carbonyl region of [4-^{13}C]Asp-labeled BR taken at −10 °C with differences near 173 and 176 ppm. The peak at 172.9 ppm shifts to 173.3 ppm after light adaption and represents the influence of chromophore isomerization on Asp_{85}. A second effect on a carboxyl group is reflected in an intensity change near 176 ppm.

spectra of BR_{la} and BR_{da} differ at two positions. Of the two resonances near 173 ppm present in dark-adapted BR, the upfield peak decreases and the lowfield peak increases upon light adaption, leading to a differential band in the difference spectrum. A weak shoulder at 172.9 ppm remains visible in the spectrum of BR_{la}. The effect can be reversed by dark-adapting the sample and could be reproduced with different

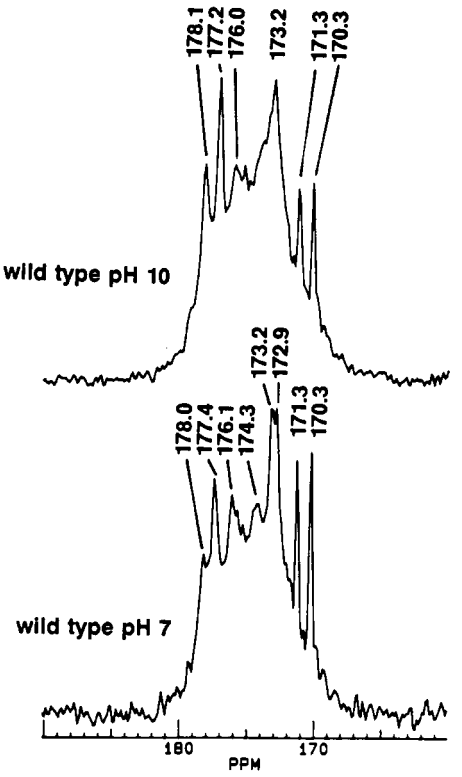

FIGURE 4: Carbonyl region of [4-^{13}C]Asp-labeled BR_{da} at pH 7 compared to pH 10 of the same sample.

samples. An intensity change also appears at 176.2 ppm, although no differential band can be found in the difference spectrum (Figure 3). The two signals at 171.3 and 170.3 ppm, assigned to protonated Asp_{96} and Asp_{115}, respectively, are not significantly affected. The small negative band at 170.3 ppm can be attributed to experimental uncertainties in intensity not being reproduced in different experiments. On the other hand, the described reproducible effects near 173 and 176 ppm are clearly beyond such errors. No significant change of Trp signals was detected.

A similar influence on resonances was observed by comparing the spectra of dark-adapted purple membrane at pH 7 and 10. Whereas the position of the peaks at 170.3 and 171.3 ppm remains unchanged at pH 10, the doublet at 173.3/172.9 ppm fuses to a single peak with σ_{iso} 173.2 ppm (Figure 4).

Relaxation. We recorded a series of CP-MAS inversion–recovery experiments with delay times between 50 ms and 8 s. As previously reported (Harbison et al., 1985), the aliphatic signals show a short relaxation time (150 ms). Figure 5 shows the relaxation behavior of the Asp and Trp bands. From this it is clear that T_1 of these groups is on the order of several seconds. The Asp signal at 178.0 ppm exhibits the fastest relaxation. After 8 s it is negative while all other resonances are still positive and the broad band of naturally abundant ^{13}C in the peptide backbone equals approximately zero. Thus, the method allows the suppression of the signal of the abundant ^{13}C backbone nuclei, and groups with faster and slower relaxation as compared to the backbone can be discerned. The relaxation times of the groups between 170 and 176 ppm are longer compared to those of the two signals downfield of 176 ppm.

Also the [11-^{13}C]Trp signals show different relaxation times, although the variation is not as large as for the Asp residues. The peak at 112.8 ppm decays fastest and equals zero after about 6 s. The next slower signal (band at 111.9 ppm) crosses zero after 8 s. The intense band at 110.6 ppm with shoulder

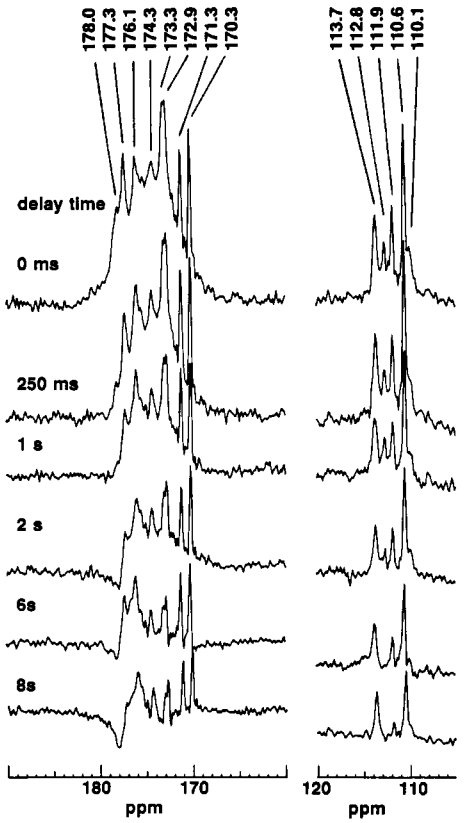

FIGURE 5: CP inversion–recovery series showing the [4-^{13}C]Asp and [11-^{13}C]Trp peaks of BR for delay times as indicated.

at 110.1 ppm as well as the lowfield signal at 113.7 ppm show considerably slower relaxation, similar to that of the Asp peaks at 170.3 and 171.3 ppm.

Spectrum without Cross Polarization. Without the use of cross polarization, the relative signal intensities vary drastically compared to the CP spectra. In addition, the positions of the detected sharp resonances differ (Figure 6).[2] Without CP and a recycle delay of 5 s, the signals downfield of 176 ppm are stronger. The peaks at 170.3, 171.3, and 173.3/172.9 are missing (the two shoulders may represent the residual bands), and the band at 176.1 ppm is very weak. Sharp peaks at 175.5, 174.9, 173.6, and 172.6 ppm now become visible which are otherwise hidden under the signals arising from CP. Although the differences are very small, bands with σ_{iso} deviating from those in the spectra taken with CP must be caused by different groups (see discussion). The contribution of the peptide backbone signal is significantly smaller, and the Trp signals are not detected.

Discussion

There are four Asp's in the interior part of BR (Asp_{85}, Asp_{96}, Asp_{115}, and Asp_{212}), four Asp's in the loops, and one Asp in the C-terminus. Our previous work with BR mutants assigned the signal at 171.3 ppm to protonated Asp_{96}. From its upfield position, we concluded that the signal at 170.3 ppm also represents a protonated Asp. Since from FTIR experiments on mutants a second protonated carboxyl group was identified with Asp_{115} (Braiman et al., 1988), we assigned this band to

[2] The spectrum in Figure 6a shows a much better resolution as compared to non-CP spectra we reported previously (Englehard et al., 1989). We have no clear explanation for these differences, which might have been due to spectrometer adjustments and/or sample preparation. The resolved resonances of Figure 6a could be reproduced with different samples.

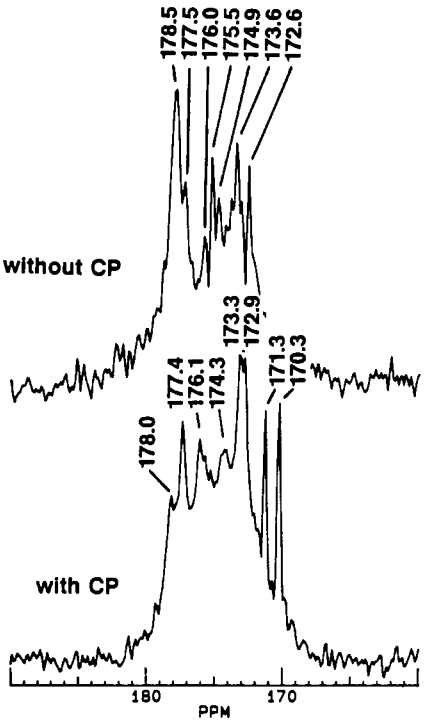

FIGURE 6: Carbonyl region of spectra of [4-^{13}]Asp-labeled BR_{da} taken with and without cross polarization.

Asp_{115}. The peak(s) near 173 ppm were tentatively assigned to Asp_{85}, but the resonance of Asp_{212} was not yet identified. To evaluate the CSA in molecular terms, some general remarks have to be made.

The width of the chemical shift anisotropies for carboxyl groups is on the order of 140 ppm (Griffin et al., 1975; Pines et al., 1974). The elements of the carboxyl tensor are similar in different molecules, and the average values are reported to be σ_{11} = 245 ppm, σ_{22} = 177 ppm, and σ_{33} = 105 ppm (Veeman, 1984). The principal axes of σ_{11} and σ_{22} are oriented in the carboxyl plane. For a symmetric unprotonated group, the axis of σ_{11} forms a bisection between the two oxygen bonds. The direction of σ_{11} and σ_{22} and the value of σ_{22} are most sensitive for protonation and hydrogen bonding. A rotation of the σ_{11} and σ_{22} axes around σ_{33} occurs upon protonation of the carboxyl group. Although protonation of a carboxyl group always results in an upfield shift of σ_{22}, the comparison of the CSA of carboxyl groups in different molecules shows that the absolute value of σ_{22} may not be taken as an unambiguous indication for the protonation state. For example, in oxalates, most likely due to interaction of the close carboxyl groups, σ_{22} is found about 25 ppm upfield of the cited average value even for a deprotonated group (Griffin et al., 1975). But again, protonation leads to an additional upfield shift of σ_{22}. In the dipeptides we examined (Table II), we find an upfield shift larger than 20 ppm for σ_{22} of the protonated carboxyl group in Asp as compared to the nonprotonated C-terminal group.

The most important information contained in Figure 2 is the relative values of the principal tensor elements. The observation of an upfield shift of σ_{22} for the resonances at 170.3 and 171.3 ppm (Asp_{96} and Asp_{115}) clearly identifies them as protonated internal Asp carboxyl groups in the BR initial state. This is also true for BR at pH 10 because no significant change in shift anisotropy occurs at high pH. The very high value of σ_{22} argues for a nonpolar environment, because it is known that hydrogen bonding would decrease the shielding of σ_{22} (Griffin et al., 1975). The value of σ_{22} near 180 ppm indicates that the two other internal aspartic acids are deprotonated. In principle, strong hydrogen bonding involving simultaneously

a strong donor and acceptor can make the two C–O bonds more equivalent. This results in a σ_{22} value approaching that of carboxylates. Acetic acid, where the two C–O bonds only differ by 0.1 Å due to a hydrogen-bonded network (σ_{22} = 180 ppm), represents such an example (van Dongen-Torman et al., 1977). However, such an extreme situation is not expected for the carboxyl groups in BR. Accordingly, the protonation state of all internal Asp's in the BR initial state could be characterized. (It can be expected that all rigid, i.e., internal, groups can be seen in the cross-polarization experiments, but it could be that some very flexible external groups have so far escaped detection; see below.) It should be emphasized that the small downfield shift of σ_{33} and σ_{11} upon protonation leaves σ_{iso} similar to the value obtained by a deprotonated Asp (e.g., 172.9 ppm compared to 171.3 ppm), and thus σ_{iso} is not an unambiguous indicator for the protonation state. Our results demonstrate that the evaluation of the full CSA tensor is a necessary tool for the determination of the protonation state of carboxyl groups in a large protein.

The CSA widths of 125 ppm (Figure 2) are close to those found for crystalline amino acids (140 ppm) (Haberkorn et al., 1981; Naito et al., 1981). For the carboxyl group of the dipeptide Ala–Asp, we found a widths of 138 and 145 ppm for the backbone peptide carbonyl of native BR (data not shown). Motion can partly average the shift anisotropy, and thus the total width of the CSA is sensitive to mobility. Our results argue for a rigid environment for the internal Asp. In contrast, the resonance at 178.5 ppm shows a considerably smaller CSA width of about 70 ppm which is explained by a greater mobility of this side chain. σ_{11} and σ_{22} shift to higher field and σ_{33} approaches σ_{22}. The high value of σ_{iso} indicates a deprotonated group. A rotational movement about the C–C axes of the side chain (i.e., about the direction of σ_{11}) could explain the changes in σ_{22} and σ_{33} with σ_{11} less affected for a symmetric carboxyl group. Hydrogen bonding can change the orientation of the tensor in the carboxyl plane, and thus the motion around C–C would also affect σ_{11} as observed. However, in case of a symmetric group, a more complex movement has to be assumed to explain the shifts of the tensor elements.

The effect of light and dark adaptation on internal Asp residues provides further structural information. The signals at 173.3 and 172.9 ppm are due to only one aspartic acid of which the shielding depends on the retinal geometry (all-trans vs 13-cis, 15-syn). After light adaptation (all-trans content > 90%), the intensity of 172.9 ppm decreases in favor of the signal at 173.3 ppm. A remaining weak shoulder reflects the small fraction of 13-cis containing BR. Since the doublet disappears in the spectra of a mutant in which Asp_{85} is replaced by a glutamic acid, we assigned it to Asp_{85} (Engelhard et al., 1990). The influence on a second aspartic acid can most likely be deduced from the intensity change at 176 ppm upon light adaption. If our assignment of Asp_{85} is correct, the second influenced band must be attributed to the remaining Asp_{212}. Since by light and dark adaption mainly the terminal part of the retinal is involved, the influence on two deprotonated aspartic acids places these residues in close proximity to the Schiff base. This is in agreement the model proposed by Henderson et al. (1990), in which Asp_{212} and Asp_{85} are located 4 Å below the Schiff base. The protonated Asp_{96} and Asp_{115} are not sensitive to chromophore isomerization, showing one sharp signal both in BR_{dA} and BR_{lA} with no significant change in position or intensity. Thus they are farther away from the Schiff base.

Trp residues are in close proximity to the chromophore and form part of the retinal-binding pocket (Polland et al., 1986; Rothschild et al., 1989; Henderson et al., 1990). But they are not located in the vicinity of the Schiff base. Our results on BR_{la} and BR_{da} show no difference in the Trp signals, which might be expected in analogy to the effects on internal deprotonated Asp. This suggests that the changes of the chromophore upon light and dark adaptation do not strongly affect the chemical environment of the Trp.

The resonance at 172.9 ppm, representing Asp_{85} with the chromophore in the 13-cis, 15-syn configuration, is missing at pH 10 in wild-type BR (Figure 4). Though we have no detailed explanation, an indirect effect of the pH on the proton acceptor seems likely. It is noteworthy that the center peak as well as the CSA of the two protonated Asp's remain unchanged. Therefore, in agreement with our earlier observations from FTIR experiments (Engelhard et al., 1985), it can be concluded that Asp_{96} and Asp_{115} are still protonated up to pH 10. Interestingly, high pH greatly accelerates M formation (Kalisky et al., 1981) and protonation of Asp_{85} (Siebert et al., unpublished results). The changes seen in the high pH NMR spectrum could reflect a different interaction of Asp_{85} with the Schiff base, which enables a faster proton transfer.

Some remarks should be made on the relative signal intensities. When the spectra of Figures 3 (middle), 4 (bottom), and 6 (bottom) are compared, differences in signal intensities were found, although all spectra were taken of BR_{da} at neutral pH. We found that in general the relative intensities of the signals attributed to internal Asp and Trp are less affected compared to the signals below 176 ppm; their intensities varied with different samples or even for the same sample at different times. So we compare relative intensity changes only within spectra of the same sample taken at short intervals, as in the case of the light-adaption experiments. We attribute the differences in part to spectrometer adjustments such as the matched Hartmann–Hahn condition for cross polarization. Probably the sample preparation also plays a role. Such factors, especially the state of aggregation of the purple membranes, will affect predominantly the external part of the protein. Therefore, it can be expected that such sample conditions, which are not easily kept under control in an NMR experiment, will influence the spectra and can explain in part the intensity variations.

The observed CSA widths indicate that one aspartic acid (178.5 ppm) is appreciably more mobile than the other residues. This should also be reflected in T_1 measurements (Figure 5). In fact, the signal at 178.0 ppm has the fastest longitudinal relaxation time (<5 s). It should be mentioned that the relaxation time of this peak is shorter than that of the backbone signal, while all other signals relax slower. In agreement with the much less but still discernibly reduced CSA width of the resonance at 177.5 ppm, this signal exhibits the next fastest relaxation. The signals at 170.3 and 171.3 ppm of the internal Asp_{96} and Asp_{115} show the slowest decrease in the inversion–recovery experiment (Figure 5). The split band at 173 ppm assigned to Asp_{85} and the peak at 176 ppm also have a long relaxation time. Thus, an additional argument is presented for the assignment of the signal at 176 ppm to the fourth internal Asp. Some uncertainty remains about the origin of the band at 174.3 ppm, which becomes well resolved at long delay times. We found this rather broad signal already in our earlier studies though with varying intensities. It is not affected upon light adaption. The long relaxation time argues for a rigid environment of this group(s). After subtraction of the broad naturally abundant carbon peak, which has its maximum

at approximately 174 ppm (Figure 1), the signal is hardly discernible. Reliable evaluation of the CSA could therefore not be undertaken.

Cross polarization should be most effective for internal Asp's due to their rigid environment, whereas experiments without CP and a short recycle delay should favor mobile groups. In accordance with the assignments we have made so far, the internal Asp's have reduced intensities or are not detected without CP and with a 5-s recycle delay. The resonances at 170.3 and 171.3 ppm (Asp_{96} and Asp_{115}) and the doublet at 173.4/172.9 ppm are missing. The peak at 176 ppm (as found in the CP spectra) is much smaller, which again argues for an internal Asp. The resonances below 176 ppm show short T_1 and smaller CSA widths and have been, therefore, attributed to external mobile groups. In agreement with this, they lead to strong peaks in the non-CP spectrum. New sharp peaks arise without CP. Though the differences in σ_{iso} are very small compared to the CP spectra, the effects are reproducible. In general, while intensities vary depending on spectrometer adjustment and other experimental conditions, the isotropic chemical shift is reproduced within the experimental resolution (0.3 ppm). Aliphatic bands can be taken as an internal standard. The new peaks without CP could arise from label that, to a small extent, scrambled to other groups than Asp (Englehard et al., 1989). Especially some incorporation into the C-1 position of glutamic acid and glutamine is observed. Since, according to the model derived by Henderson, these groups are located in the longer loops at the periphery of the protein, they are probably rather mobile. This would explain the appearance of the resonances in the non-CP spectra and their small contribution in the normal CP experiments.

The Trp resonances at 113.7 and 110.6 ppm have relaxation times comparable to the protonated Asp, while the two remaining bands decrease significantly faster (Figure 5). The fastest relaxation at 112.8 ppm is in agreement with the observation of a smaller CSA for this Trp band. Although all eight Trp residues in BR are placed in the internal protein region (Henderson et al., 1990; Soppa & Oesterhelt, 1989) some difference in side-chain mobility seems to exist.

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Water Structural Changes in the Bacteriorhodopsin Photocycle: Analysis by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The Fourier transform infrared difference spectra between light-adapted bacteriorhodopsin (BR) and its photointermediates, L and M, were analyzed for the 3750-3450-cm^{-1} region. The O-H stretching vibrational bands were identified from spectra upon substitution with ^2H_2O. Among them, the 3642-cm^{-1} band of BR was assigned to water by substitution with $H_2{}^{18}O$. By a comparison with the published infrared spectra of the water in model systems [Mohr, S. C., Wilk, W. D., & Barrow, G. M. (1965) *J. Am. Chem. Soc. 87*, 3048-3052], it is shown that the O-H bonds of the water in BR interact very weakly. Upon formation of L, the interaction becomes stronger. The O-H bonds of the protein side chain undergo similar changes. On the other hand, M formation further weakens the interaction of the same water molecules in BR. The appearance of a sharp band at 3486 cm^{-1}, which was assigned tentatively to the N-H stretching vibration of the peptide bond, is unique to L. The results suggest that the water molecules are involved in the perturbation of Asp-96 in the L intermediate and that they are exerted from the protonated Schiff base which changes position upon the light-induced reaction.

Bacteriorhodopsin (bR)[1] is an intrinsic membrane protein which transports protons upon absorption of light by its retinylidene chromophore (Stoeckenius et al., 1979; Stoeckenius & Bogomolni, 1984; Mathies et al., 1991). In the transport process, protons are released to the exterior side of the membrane at the same time or just after the conversion of an L intermediate to an M intermediate, in which the Schiff base is unprotonated (Drachev et al., 1984; Grzesiek & Dencher, 1986; Vârô & Lanyi, 1990; Liu, 1990; Liu et al., 1990). The deprotonation of the Schiff base facilitates the uptake of a proton from the opposite side of the membrane via Asp-96 (Holz et al., 1989; Otto et al., 1989). Thus, the structural analysis of the L intermediate is crucial for elucidating the mechanism of proton pumping.

Water has been proposed to be a molecule involved in the hydrogen-bonding network surrounding the Schiff base in the resting *all-trans*-bR (Hildebrandt & Stockburger, 1984; Baasov et al, 1987; de Groot et al., 1989; Papadopoulos et al., 1990), whose Schiff base N-H bond points toward Asp-85 and Asp-212 (Lin & Mathies, 1989). Upon isomerization, the N-H bond turns to the opposite side, toward Asp-96, because Asp-96 is perturbed for L and N but not for M, suggesting a strong interaction between the protonated Schiff base of the L or N intermediate with Asp-96 (Pfefferlê et al., 1991). However, the 1.0-1.2-nm distance between the Schiff base and Asp-96 (Henderson et al., 1990) is too far to account for the perturbation of Asp-96 by direct interaction. Serine or threonine residues (Marti et al., 1991) or water between the Schiff base and Asp-96 may form a hydrogen-bonding network, which facilitates their interaction. Henderson et al. (1990) have suggested the presence of Thr-89 and -90 and at least one or two water molecules in the hydrophobic channel between the Schiff base and Asp-96.

Except for the N-H stretching vibration of the Schiff base by resonance Raman spectroscopy (Hildebrandt & Stockburger, 1984) and a very recent study on the O-H stretching vibration of the protein side chains of bR (Chang et al., 1991), exploration of the vibrational bands in the region above 3000 cm^{-1} has never been reported for retinoid proteins. Changes in the water band in the O-H bending vibrational region have previously been observed upon formation of the bathorhodopsin form of the visual pigment (Ganter et al., 1988).

The present study aims at analyzing the O-H stretching vibrational bands in the 3800-3000-cm^{-1} region of the FTIR difference spectra of intermediates L and M to examine the vibrational bands related to the water molecules. Changes in the water bands were observed for these intermediates. The

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

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