Magic Angle Sample Spinning ^{13}C Nuclear Magnetic Resonance of Isotopically Labeled Bacteriorhodopsin†

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ABSTRACT: Bacteriorhodopsin (bR), the light-driven proton pump protein from *Halobacterium halobium*, was biosynthetically labeled with [4-^{13}C]Asp. The incorporation yield was 48%. The magic angle sample spinning (MASS) ^{13}C nuclear magnetic resonance (NMR) spectrum of this sample revealed six different peaks superimposed on a broad band of naturally abundant peptide-bond ^{13}C. Two of the six carbonyl signals can be attributed to internal-protonated Asp carboxyl groups, one of which might be Asp^{115}. An additional resonance at 110 ppm can be associated with the C-11 carbon of Trp, indicating an unusual biosynthetic pathway of this amino acid in *Halobacterium halobium*. Similar measurements performed on papain-treated purple membrane which lacks the C-terminal tail display two new intense signals at 178 and 178.9 ppm. If the same spectrum is taken without cross-polarization, these signals do not decrease or disappear. On the basis of their intensities and their chemical shifts, one can assign in addition to the C-terminal Asp four Asp residues facing the cytoplasmic phase. In native bR, at least two of these form a salt-bridge-like bond which also might include the C-terminal tail. These experiments not only provide data about the chemical environment of the Asp residues within the hydrophobic core of bacteriorhodopsin but also yield information about the interactions between surface components.

The light-driven intramolecular proton transfer in bacteriorhodopsin (bR)[1] the only protein of the purple membrane from *Halobacterium* halobium [for recent reviews, see Ottolenghi (1980), Ovchinnikov et al. (1982), Hess et al. (1982), Stoeckenius and Bogomolni (1982), and Dencher (1983)], is activated by the reversible photoisomerization of the retinal chromophore. Several mechanisms of proton translocation were proposed which rely either on hydrogen-bonded chains (Nagle & Tristam-Nagle, 1983; Merz & Zundel, 1981) or on displacement reactions from one defined site to the next one (Hess et al., 1982; Engelhard et al., 1985; Engelhard & Hess, 1987). There are only a few charged amino acids within the hydrophobic core of bR (Trewhella et al., 1983) which might take part in the proton translocation. It was shown by Fourier-transform infrared (FTIR) techniques that tyrosine vibrations were altered during the photocycle, and these data were interpreted as evidence of protonation changes of specific tyrosines (Dollinger et al., 1986; Roepe et al., 1987). Protonation and deprotonation of carboxyl groups were first described by Rothschild et al. (1981), Siebert et al. (1982), and Bagley et al. (1982). The use of isotope-labeled aspartic acid incorporated biosynthetically into bR identified these groups as aspartyl residues (Engelhard et al., 1985; Eisenstein et al., 1987). Additionally, it was shown by time-resolved infrared (IR) spectroscopy that at least two deprotonated and one protonated aspartic acid take part in the photoreaction cycle, leading to the suggestion that these particular amino acids are members of the proton translocation pathway (Engelhard et al., 1985). These aspartic acids are located within the hydrophobic interior of the protein. A fourth aspartic acid which might be present in the protonated (Eisenstein et al., 1987) or deprotonated state (Engelhard et al., 1985) was also located inaccessible to the bulk phase. These assignments are in accordance with structural models (Trewhella et al., 1983; Khorana et al., 1979; Ovchinnikov et al., 1979) which also place four Asp (Asp^{85}, Asp^{96}, Asp^{115}, and Asp^{212}) into the helical region and five Asp on the surface of the membrane protein. Recent experiments using site-directed mutagenesis verified these deductions (Mogi et al., 1988; Khorana, 1988) and showed that Asp^{85} and Asp^{96}, which are in the interior of the protein, are important for the function of the proton pump.

Infrared spectroscopy is specifically suitable to study kinetic and static differences of well-defined intermediates. It provides data only for molecular groups or residues which are changing their environment or chemical nature during a reaction. Very often these groups will also be of functional importance. However, for a better understanding of the function, it is necessary to obtain data of specific residues in the ground state and the intermediate states. Nuclear magnetic resonance (NMR) spectroscopy is principally capable to provide such detailed information. Moreover, the solution structure of small proteins can be gained by this method. However, the elucidation of the conformation and the chemical environment of specific amino acids in large proteins and membrane proteins in particular is hampered by the fact that high-resolution methods cannot be applied because of severe line broadening. Making use of recent developments, solid-state nuclear mag-

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[1] Abbreviations: CP, cross-polarization; bR, bacteriorhodopsin; FTIR, Fourier-transform infrared; FID, free induction decay; IR, infrared; MASS, magic angle sample spinning; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; TCA cycle, citric acid cycle; TFA, trifluoroacetic acid; TMS, tetramethylsilane.

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netic resonance [see Fyfe (1983) and references cited therein] in combination with isotope enrichment of functional groups allowed the elucidation also of molecules with high molecular weights and restricted mobility. These experiments make use of "dilute" nuclear spin systems which allow the observation of simplified spectra with negligible homonuclear dipolar interactions. The heteronuclear dipolar interactions with the proton can be removed by appropriate decoupling fields. This leaves only the chemical shift anisotropy as a dominant, line-broadening effect.

The solid-state NMR technique was in the case of biological systems especially applied to bacteriorhodopsin. The dynamic properties of specific side chain functions of this two-dimensional, crystalline membrane protein were studied by using ^2H NMR (Keniry et al., 1984; Herzfeld et al., 1987). ^{13}C NMR could demonstrate a relatively rigid protein backbone (Spohn & Kimmich, 1983). Furthermore, the solid-state technique was applied to questions of the secondary structure of the bR molecule using the line shape of the powder spectrum (Lewis et al., 1985). More detailed information about the conformation, the chemical environment, and the isomerization state of the retinal chromophore was gained by the incorporation of a specific isotope label into the chromophore and the protein (Harbison et al., 1984a,b, 1985). A similar approach was also applied to the visual pigment bovine rhodopsin (Mollevanger et al., 1987; Smith et al., 1987). These examples show that NMR spectroscopy not only is capable of solving specific problems but also can be understood to complement other methods, especially infrared spectroscopy.

Further insight into the structural and chemical properties of specific amino acids in bacteriorhodopsin was obtained from the solid-state NMR experiments of [4-^{13}C]Asp–bR and model compounds which we describe here.

Materials and Methods

All reagents used were reagent grade. The peptides Ala-Asp, Gly-Asn-Ala, and Gly-Gly-Asn-Ala were bought from Bachem, Bubendorf, Switzerland, DL-[4-^{13}C]Asp (99% ^{13}C) and L-[4-^{14}C]Asp (2 mCi/mmol) were purchased from Amersham Buchler, Braunschweig.

The bacteria were grown in a synthetic medium as described by Onishi et al. (1965) which was modified according to Engelhard et al. (1985). The ^{13}C-labeled aspartic acid replaced the unlabeled Asp in the medium. Bacteriorhodopsin was isolated from *Halobacterium halobium* according to Oesterhelt and Stoeckenius (1974). The last purification step was a sucrose density gradient as described by Oesterhelt and Stoeckenius (1974). The incorporation was determined by a radioactive tracer, [4-^{14}C]Asp, as described by Engelhard et al. (1985). The incorporation yield was also evaluated by IR techniques (Engelhard et al., 1985) and was calculated to be 48%, which is in agreement with the results obtained from the incorporation of [4-^{14}C]Asp.

Bacteriorhodopsin was hydrolyzed in 6 N HCl or, for the determination of Trp, in TFA–thioglycolic acid–propionic acid according to Yokote et al. (1986) and subsequently analyzed on a Biotronik Model 7000 amino acid analyzer. In a separate run, the fractions from the amino acid analyzer were collected, and their radioactivity was determined by using a liquid scintillation counter (Mark III; Searle Analytic Inc., Des Moines, IA). Lipids were extracted from purple membrane according to the method of Kates et al. (1982). The nonpolar lipid fraction was extracted from bleached and lyophilized purple membrane with toluene.

The C-terminal tail of bR was cleaved off by papain by using the method of Renthal et al. (1983), and the product was then analyzed by SDS–polyacrylamide electrophoresis. It should be noted that the SDS gel electrophoresis of this sample shows only one band with an apparent molecular weight slightly smaller than that of untreated native bR. It is also clear from the protocol of the purification of the papain-treated bR that the protease and the C-terminal amino acids were separated from the membrane.

The ^{13}C solid-state NMR measurements were performed on a Bruker CXP 300 NMR spectrometer (Bruker GmbH, Karlsruhe) operating at 75.5 MHz for ^{13}C and at 300 MHz for ^1H (7.1 T). Approximately 40–50 mg of bR lyophilized at 0.1 Torr was filled into a cylindrical rotor made of aluminum oxide. The pH of the samples was determined before lyophilization. The native and the papain-treated bR suspension in distilled water had a pH of 6 whereas the blue membrane had a pH of 4.5. The samples were spun at the magic angle in the double-bearing wide-bore system of the Bruker probe head at spinning speeds between 2.7 and 2.8 kHz. The spectra were recorded from approximately 50 mg of dry or humidified samples. Hydrated bR was obtained by adding 80–100 wt% of water into the MASS rotor. The temperature of the bearing nitrogen gas was regulated and kept constant by a home-built heat exchanger. All spectra were taken at room temperature. The chemical shifts are relative to external tetramethylsilane (TMS).

Spectra were measured both with and without the standard cross-polarization (CP) technique. In both cases, the FID was detected for 50 ms under proton decoupling with a frequency of 75–80 kHz. The ^{13}C spectral width was 20 kHz with a carrier frequency of 7.2 kHz corresponding to 95 ppm; the decoupler offset was set to the middle of the proton spectrum. Typically, 10^4 to 2×10^4 FID's were accumulated with 2K data points, adjusted (zero-filled) up to 4K points, processed by a 10-Hz exponential multiplication, and then Fourier-transformed. For CP spectra, the mixing time under adjusted Hartman–Hahn conditions was 1.5 ms, and the recycle delay between pulses was 5 or 10 s. The different recycle delays had no influence on the spectra. The 90° pulse time for ^1H was 3.4 μs. In order to display carbons that undergo only an unefficient polarization transfer, spectra were also recorded without cross-polarization despite the loss of sensitivity and the longer acquisition time (up to 2 days). The recycle delay between pulses was varied in the range of 5–60 s.

To distinguish between hydrogen-bonded and non-hydrogen-bonded unsaturated carbons, the pulse sequence of Opella and Frey (1979) was used. After the mixing time in the normal CP sequence, a 40-μs delay without proton decoupling was inserted.

To improve resolution of overalpping resonances, the FID was multiplied by the two-term exponential function $\exp(-at - bt^2)$ where $a = \pi(40\text{ Hz})$ and $b = -a/\text{AQ}$ (AQ = acquisition time) (Ferrige & Lindon, 1978). This procedure will result in a Gaussian line shape for resonances with approximately 40-Hz (corresponding to 0.5 ppm) half-height line width.

Results

Distribution of the ^{14}C Label. The incorporation of [4-^{14}C]Asp into bR and the scrambling of the ^{14}C isotope into other amino acids as well as the polar and unpolar lipids are shown in Table I. Besides aspartic acid, a substantial amount of radioactivity was also found in glutamic acid and tryptophan. The incorporation of the label into Glu reflects the biosynthetic connection of Asp and Glu through the tricarboxylic acid (TCA) cycle. Due to the stereospecificity of the corresponding enzyme reactions, the label from the side chain carboxyl group of Asp is transferred to the α-carboxyl

Table I: Metabolic Destiny of the ^{14}C Label in [4-^{14}C]Asp[a]

amino acid	position of label	biosynthetic group	incorporation yield
Asp, Asn	γ-carboxyl	oxalacetate	48
Glu, Gln	α-carboxyl	α-ketoglutarate	20
Thr	side chain	oxalacetate	<3
Gly?, Ala?	side chain	3-phosphoglycerate pyruvate	<3
Tyr	aromatic ring	erythrose 4-phosphate	<3
Phe	aromatic ring	erythrose 4-phosphate	<3
Trp	C-11	erythrose 4-phosphate	≈50–100

[a] The incorporation yield was determined after acid hydrolysis of bR by analyzing the specific activity of the amino acids divided by the specific activity of the originally used Asp. It could not be distinguished between Gly and Ala because they were eluting very close to each other. Only an approximate incorporation yield of radioactivity into Trp could be determined because of a very poor recovery of Trp after hydrolysis.

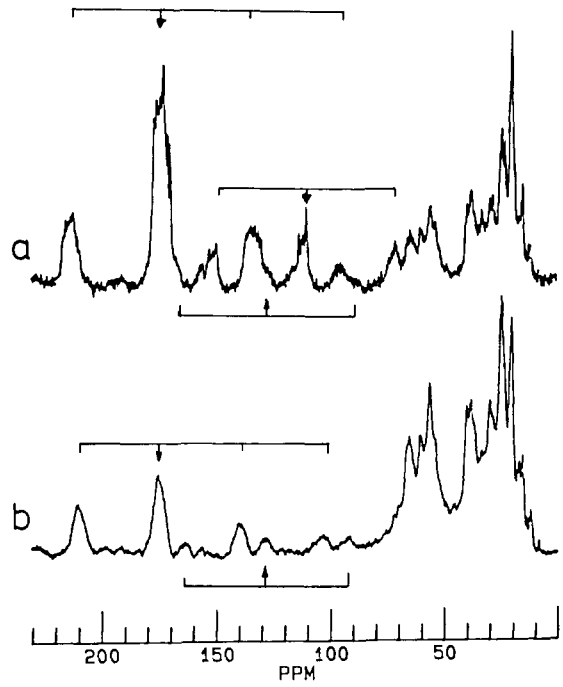

FIGURE 1: ^{13}C MASS NMR spectra of (a) [4-^{13}C]Asp–bR and (b) native bR. (a) Recycle delay, 10 s; spinning speed ν_{rot}, 2950 Hz; (b) recycle delay, 5 s; ν_{rot}, 2700 Hz. The spinning side bands are marked by bars and the isotropic resonances with an arrow. The spectra were taken with the standard cross-polarization technique (mixing time 1.5 ms) under ^1H broad-band decoupling.

of Glu. The relatively high amount of radioactivity found in Trp cannot be accounted for by the usual bacterial biosynthetic pathways. The label could also be determined to a minor extent in the polar and unpolar lipid fractions as well as in retinal, but neither a quantitative analysis nor further investigations in this respect were so far undertaken.

General Resonances. The solid-state ^{13}C NMR of the [4-^{13}C]Asp-modified purple membrane (Figure 1) shows pronounced resonances resembling those of the native bR (Spohn & Kimmich, 1983; Harbison et al., 1985) with centers at 176 ppm for the carbonyl, at 130 ppm for the aromatic, and between 45 and 80 ppm and between 10 and 40 ppm for the aliphatic ^{13}C nuclei (ppm relative to TMS). An additional band is observed between 109 and 114 ppm with sharp resonances at 110.5, 112, and 114 ppm (Figure 2, trace a), indicating quite clearly that during the growth of the bacteria Asp is used for the biosynthesis of metabolites. The chemical shift range is characteristic for aromatic, acetal, and sp^2 carbons (Kalinowski et al., 1984). When the chemical shift is compared with data from the literature, only the C-4 (108.8

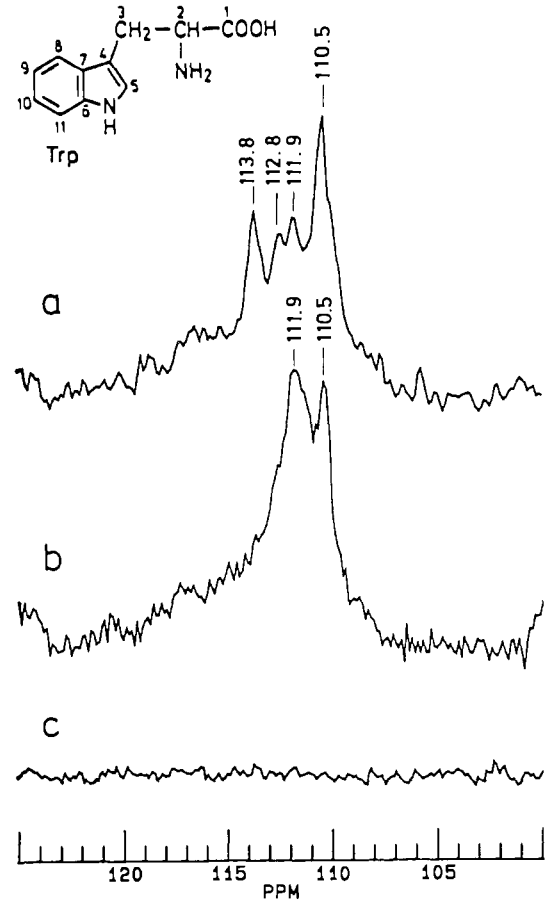

FIGURE 2: Aromatic region of ^{13}C MASS NMR spectra of (a) [4-^{13}C]Asp–bR and (b) the corresponding blue membrane. The recycle delay was 10 s in (a) and 5 s in (b). (c) Same as (a), but with a 40-μs delay after CP to suppress the signals of unsaturated carbons with bonded hydrogens.

ppm) and C-11 (113.3) carbons of the Trp aromatic ring system (Horwarth & Lilley, 1978) or the C-14 of retinal in bR[548] (110.5 ppm; Harbison et al., 1985) can be responsible for these resonances. Control experiments using FTIR difference spectroscopy showed that the latter source is unprobable because no isotope shift of the retinal vibrational modes could be detected. Therefore, the NMR signal around 112 ppm can be attributed to Trp residues, confirming the results of the ^{14}C incorporation into Trp. The aromatic carbon at C-11 is bound to a hydrogen whereas the C-4 position has only carbon as neighbors. To distinguish between these two possibilities, a pulse sequence with a 40-μs delay between CP and acquisition was used, which suppresses the signals of hydrogen-bonded carbons (Opella & Frey, 1979). Under these conditions, the Trp signals disappeared completely (Figure 2, trace c), indicating that only the C-11 carbon is responsible for these resonances. It is now possible to attribute the bands to five to six different chemical environments of Trp residues in bR. If the NMR spectrum is taken without cross-polarization (data not shown), these resonances become very small, indicating a rigid fixation of the aromatic ring of Trp. It should be added that this spectral area is modified in the blue membrane (Figure 2, trace b) which reflects the altered chemical environment of Trp residues within the hydrophobic core of the protein.

Carbonyl Resonances of Model Compounds. In Figure 3, the spectra of the carbonyl region of three peptides with the sequences Ala-Asp, Gly-Asn-Ala, and Gly-Gly-Asn-Ala are shown. The assignments are summarized in Table II. The resonances of the protonated and deprotonated carboxyl groups

Table II: Solid-State ^{13}C Chemical Shift Parameters of Carbonyl Resonances from the Peptides Ala-Asp, Gly-Asn-Ala, and Gly-Gly-Asn-Ala[a]

sample	chemical shift (ppm)			
	-COO⁻	-COOH	-$CONH_2$	-CONH-
Ala-Asp	178.4 (1, s)	174.5 (1, s)		172.5 (1, d)
Gly-Asn-Ala	179.0 (1, s)		175.7*	175.7*, 168.4 (1, d)
Gly-Gly-Asn-Ala	180.0 (1, s)		175.5 (1, d)	172.0 (1, d), 168.0 (2, m)

[a] The assignments were made by using the data from Grathwohl et al. (1974). The number of carbons and the multiplet character of the signals are given in parentheses (s, singlet; d, doublet; m, unresolved multiplet). Asterisks indicate that one peptide bond and also the γ-carboxyl of Asn are absorbing at the same position (see Figure 3).

Table III: ^{13}C Chemical Shift Parameters of Resonances Taken from Figures 3–5

resonance	chemical shift (ppm)		
	bR(native)	bR^{blue}	bR^{pap}
A1	170.2	170.5	170.2
A2	171.2	170.9	171.2
A3	173.0		172.9
A4	174.2		
A5	175.8		
A6	177.2		
B1		175.0	
B2		177.8	
C1			177.2
C2			178.0
C3			178.9

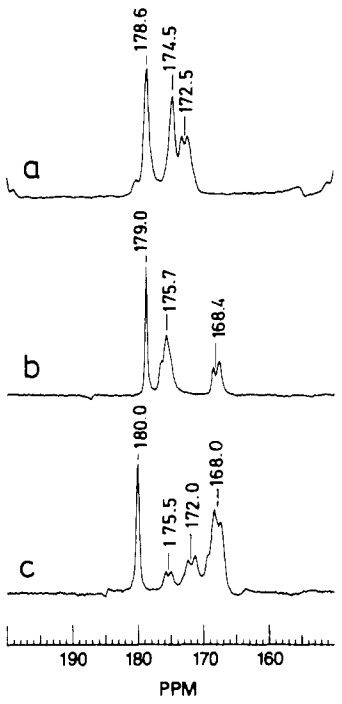

FIGURE 3: ^{13}C MASS NMR spectra of the model compounds (a) Ala-Asp, (b) Gly-Asn-Ala, and (c) Gly-Gly-Asn-Ala. The spectra of the natural-abundancy ^{13}C carbons were taken with cross-polarization [mixing time, 1.5 ms; recycle delay, 5 s for (b) and (c); in (a), the mixing time was 3 ms, and the recycle delay was 10 s].

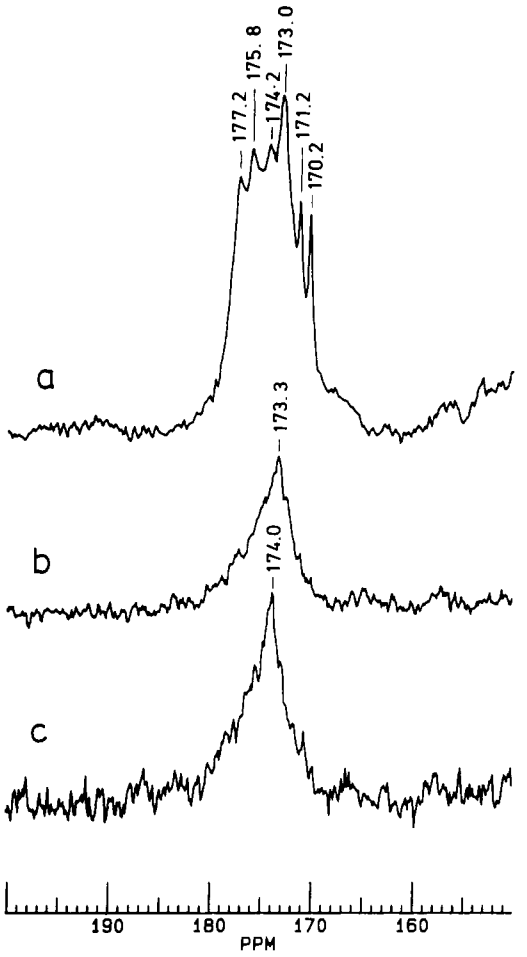

FIGURE 4: ^{13}C MASS NMR spectra of [4-^{13}C]Asp-bR in the carbonyl region taken (a) with cross-polarization (mixing time 1.5 ms) and (b and c) without cross-polarization. The recycle delay was 10 s in (a), 5 s in (b), and 60 s in (c).

appear as sharp resonances (half-width <0.5 ppm) above 174 ppm. The resonance for the deprotonated carboxyl group in Ala-Asp is located at 178.4 ppm whereas the protonated carboxyl group is observed at 174.5 ppm. The amide carbon of Asn has its resonance as a doublet at 175.5 ppm, whereas the chemical shifts of the peptide bonds vary between 176 and 168 ppm depending on the particular amino acid sequence. The amide resonances of Asn and the peptide-bond carbon are doublets with a much lower amplitude than the carboxyl signals. This broadening effect is due to the quadrupolar coupling of the ^{14}N nucleus with the ^{13}C carbon. Similar effects were already described by several authors [e.g., see Groombridge et al. (1980)] and theoretically evaluated by Hexem et al. (1981).

Carbonyl Resonances in Native bR. The chemical shift regions of the carboxyl and amide carbons from Asp, Asn, and Glu are expected to fall between 160 and 180 ppm. The natural abundancy of ^{13}C of all amide and carboxyl groups in bR leads to a pronounced broad signal with the center at 176 ppm and a line width at half-height of about 6.5 ppm (Harbison et al., 1983; Spohn & Kimmich, 1983) (Figure 1, trace b) whereas the intensity of the isotope-labeled sample (Figure 1, trace a) is substantially increased in the high-field region of this peak and the half-height line width has gained 1 ppm.

Figure 4 (trace a) shows the carbonyl region between 150 and 200 ppm. Six well-resolved resonances are superimposed on a broad signal which comprises the naturally abundant carbonyl carbons. The spectrum was recorded by using the cross-polarization technique. Under these conditions, the signals of the carbon nuclei with neighboring hydrogens and/or

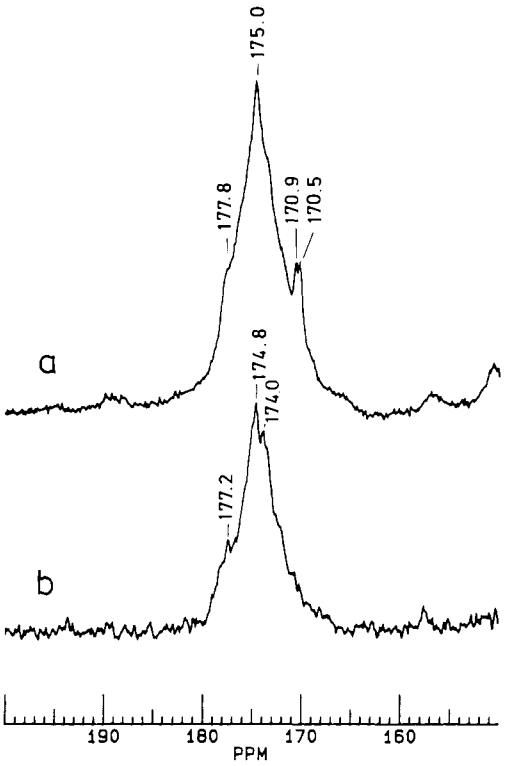

FIGURE 5: ^{13}C MASS NMR spectra of cation-depleted [4-^{13}C]Asp–bR (^{13}C-bR^{blue}) in the carbonyl region taken (a) with cross-polarization (mixing time, 1.5 ms) and a recycle delay of 5 s. The spectrum in (b) was measured without cross-polarization. The recycle delay was in this case 60 s. According to Figure 4b,c, it is not expected that the spectrum would be changed by using a recycle delay of 5 s.

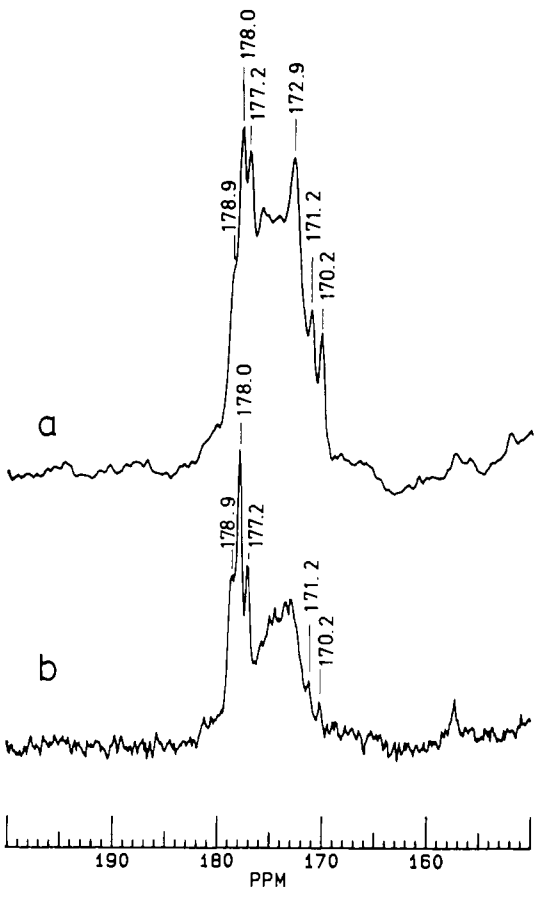

FIGURE 6: ^{13}C MASS NMR spectra of papain-treated [4-^{13}C]Asp–bR (^{13}C-bR^{pap}) in the carbonyl region taken (a) with cross-polarization (mixing time 1.5 ms) and (b) without cross-polarization. The recycle delay was 5 s in (a) and (b).

with slow molecular motion are enhanced (Alemany et al., 1983a,b). In trace b of Figure 4, the spectrum of the same sample as in trace a is recorded without cross-polarization. The recycle delay was 5 s. A recycle delay of 60 s (Figure 4, trace c) does not change the spectrum. It can be seen that the relative intensity of the two resonances furthest upfield (A1, A2) is decreased, whereas the signal at 173.3 ppm is enhanced.

Spectra were also taken from lyophilized samples (data not shown). The sharp resonances which are observed in the wet sample are not very well resolved and only visible as shoulders on a broad underlying band.

Carbonyl Resonances in bR^{blue}. The replacement of intrinsic cations of the purple membrane by protons through ion-exchange chromatography (Kimura et al., 1984) leads to the blue membrane (bR^{blue}). All exterior aspartyl residues should absorb at one position resembling hydrated protonated carboxyl groups. The NMR spectrum of this sample (Figure 5) displays a prominent resonance at 175 ppm (B1). This chemical shift provides a reference position for external protonated carboxylates in this system. On the high-field edge of this band, the two sharp resonances are still visible but with altered chemical shifts. The small shoulder at 174 ppm might stem from the same group which gives rise to the signal at 173 ppm (A3) of Figure 4. The spectrum which was taken without cross-polarization (trace b of Figure 5) displays a smaller overall line width with two minute bands at 174 and 177.2 ppm. Because the pH of the sample was not lower than pH 4.5, the latter signal might be due to a small population of unprotonated carboxyl groups.

Carbonyl Resonances in Papain-Treated bR (bR^{pap}). To further assign the resonances to specific aspartic acids, the C-terminal tail of bR was proteolytically cleaved by papain and quantitatively separated from bR by centrifugation. Papain is known to split 16–18 amino acids from the tail including Asp^{242} (Abdulaev et al., 1978). The NMR spectra of such a sample are shown in Figure 6. Again, the three high-field resonances (A1, A2, A3) of the native sample can be identified, whereas the 174.2 ppm line (A4) is vanished and the 175.8 ppm signal (A5) only barely visible. The intensity at 177.2 ppm (C1) is enhanced. Furthermore, two new resonances appear at 178.0 ppm (C2) and 178.9 ppm (C3). Omitting the cross-polarization reveals some new features of this particular sample (trace b of Figure 6). The three low-field peaks are enlarged relative to the remaining high-field absorbance range. The resonance originally located at 172.9 ppm (A3) is reduced and visible only as a double peak with the center at 175 ppm. The other two signals at 170.2 ppm (A1) and 171.2 ppm (A2) are also diminished in their intensity as they are in the case of native bR and bR^{blue}. Under the experimental conditions maintained, the area of the low-field signals (C1, C2, C3) is approximately proportional to the number of nuclei. Deconvolution of this spectrum as described under Materials and Methods (Figure 7) supports the existence of three signals in a ratio of 1:2:1. Thus, it becomes possible to obtain the number of ^{13}C carbons responsible for the three low-field resonances (see Discussion). The spectrum of the cation-depleted bR^{pap} is almost identical (data not shown) with the native blue form (Figure 5). It can be concluded that the new resonances seen in bR^{pap} are due to four external water-exposed aspartic acids.

Discussion

In principle, the isotropic chemical shifts obtained by MASS NMR of isotopically labeled bR can be compared with the shifts of model substances and then related to the chemical environment of the corresponding nucleus in the protein.

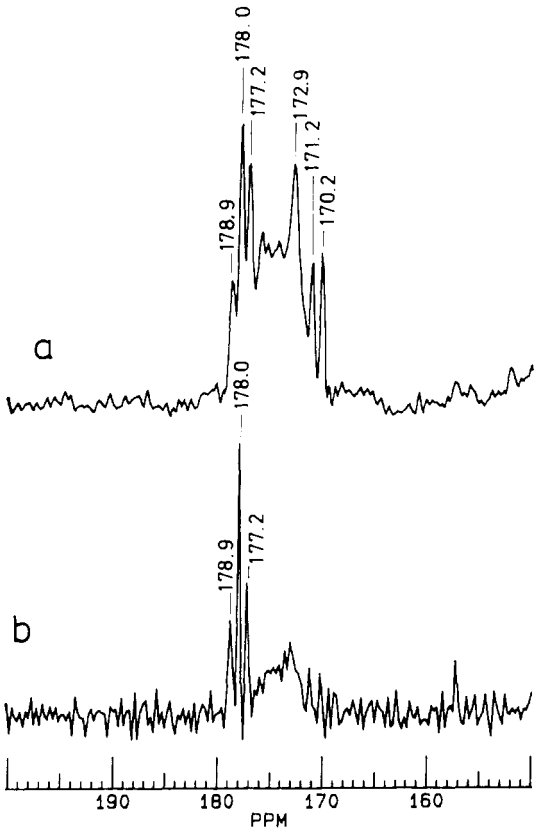

FIGURE 7: Deconvolution of the spectra of Figure 6 with a negative line broadening of 40 Hz (see Materials and Methods for further details).

However, due to the complex microenvironment of an amino acid side chain in a protein, their chemical shifts can differ drastically from the same residue when it is part of a simple peptide. For example, the CH_2– proton resonances of an arginine in the amylase inhibitor tendamistat were found 1 ppm downfield from their usual position due to the ring current effect of two adjacent aromatic amino acids (Kline et al., 1986). In addition, it should be noted that the solid-state NMR of dilute spins does not allow an assignment of resonances as compared to high-resolution proton NMR where, e.g., two-dimensional NMR techniques can be readily applied [e.g., see Kline et al. (1986)].

Another problem of assignment is the distribution of the isotope to other amino acids during biosynthesis. In earlier studies of bR, it became evident that the ^{13}C label of [4-^{13}C]Asp was found not only in Asp but also in Glu (Engelhard et al., 1985). This was to be expected because Asp and Glu are biosynthetically connected through the citric acid cycle (TCA cycle). The stereospecificity of the TCA cycle determines the fate of the isotope which emerges in the α-carboxyl group of glutamic acid. In the protein, this group will then be part of the peptide bond.

Surprisingly, the label was found not only in the Asp biosynthesis family but also to a major extent in Trp and the polar and unpolar lipid fractions (Table I). The labeling of Trp on the one hand and of the lipid fractions on the other hand yields new insight into the biosynthesis of these biomolecules in *Halobacteria* which will be investigated and discussed in detail separately. However, a few points should now be emphasized. In *Escherichia coli* bacteria, the first step of the synthesis of Trp and the other aromatic amino acids Tyr and Phe is the reaction of erythrose 4-phosphate with phosphoenolpyruvate (PEP) [for a review, see Umbarger (1978)], leading finally to chorismate. At this point, the synthesis divides into two branches, one leading to Trp and the other to Tyr and Phe. In this final reaction sequence, the second ring of Trp is introduced using phosphoribosyl pyrophosphate. Since the chemical shift of the Trp resonances and the dephasing experiment (Figure 2, trace c) both narrow the possible positions in the Trp ring system to C-11, the ^{13}C label must come from erythrose and cannot be incorporated in a later step through the ribosyl moiety. However, in contradiction to this result, only minute amounts of radioactivity were detected in the Tyr fraction, and almost no label was found in the Phe fraction. This observation could be explained by assuming that Tyr and Phe, which were constituents of the growth medium, inhibit their own biosynthesis. For a better understanding of these observations including the incorporation of the isotope into the polar and unpolar lipids, more detailed experiments on this subject are mandatory.

Eight Trp residues are contained in bR of which all but one are located at the extracellular side of the membrane (Trewhella et al., 1983). Because the label is found exclusively at one position of the Trp aromatic ring system (namely, C-11), the observed resonances can be attributed to at least five classes of Trp positioned in a different chemical environment. All Trp seem to be quite rigid on the NMR time scale because these signals are detected only with cross-polarization.

In the cation-depleted membrane (bR^{blue}), not only the chromophore displays an altered interaction with the protein as indicated by the red shift of the absorption maximum but also the side chains of Trp (Figure 2, trace b) and the interior Asp (Figure 5). Obviously, the protonation of exterior negatively charged groups which disrupts the distribution of surface charges on the membrane also has a dramatic effect on the hydrophobic core of bR.

The chemical shift of the peptide bond carbon is quite dependent on the secondary structure (Kricheldorf & Müller, 1983, 1984). For example, the signal of $(\gamma OMe\text{-}Glu)_n$ present in the form of an α-helix is found at 175.9 ppm whereas the corresponding β-sheet absorbs at 172.6 ppm (Kricheldorf & Müller, 1983). In this context, it is noteworthy that the resonance maximum of the native bR sample at 176 ppm might reflect the high degree of helical structures found in bacteriorhodopsin (Henderson & Unwin, 1975).

The resonances from Asp, Asn, and the peptide bond of Glu are also found in the carbonyl region of the MASS NMR spectrum. Assuming that all ^{13}C nuclei contribute proportionally to their concentration to the signal, then approximately 30% of the signal will stem from the naturally abundant carbon, 25% from the Glu/Gln label, and 45% from the isotope enrichment in Asp/Asn.

Theoretically, if each of the chemical environments of Asp and Asn is different, one would expect maximally 12 NMR signals between 165 and 180 ppm. On the other hand, there are at least three possible different chemical surroundings for Asp and two for Asn in a membrane protein: At pH 7, all Asp accessible to the exterior should be deprotonated. Aspartic acids within the hydrophobic core of the protein could either be protonated or be deprotonated, requiring a counterion. Since Asn is a neutral amino acid, only the hydrophobic and hydrophilic chemical environments are expected to contribute to the chemical shift positions. According to the current models mentioned above, at least four Asp are located within the hydrophobic core of the protein. One Asp contained in the C-terminal tail can be cleaved off by papain. The other four residues are members of two sequences with the general structure Asp-X-Asp, with X being Pro or Ala. They are

found in the connecting loops between helices AB and CD, respectively [the letter A, B, ..., F, G denotes the helices deduced from the amino acid sequence of bR according to Trewhella et al. (1983)]. In this case, the four Asp would not be fully hydrated but would form salt bridges, e.g., to cations. The Asn are positioned close to the surface of the membrane. These considerations amount to four to five different environments for Asp and Asn, but six different resonances are observed in bR.

A general classification of carboxyl and amide resonances can be obtained by using data from the literature and from our own solid-state NMR measurements of model substances (Figure 3). The hydrogen-bonded negatively charged carboxyl group will absorb approximately between 175 and 180 ppm. The resonance of the same group in an aprotic environment is shifted to higher fields. For example, the signal of the side chain carboxyl group of Asp shifts from 173.7 ppm in D_2O to 170.3 ppm in dimethyl sulfoxide (DMSO) (Horwarth & Lilley, 1978). A protonated carboxyl group will also be found at higher fields (Table II). The ^{13}C signal of the amide carbon of Asn is expected to be around 175 ppm (Table II).

An indication of whether the resonances of Figure 4 are due to Glu/Gln, Asn, and/or Asp might be gained by evaluating the half-widths of the resonances. The ^{14}N of the amide has a spin of 1. The quadrupolar coupling of the ^{14}N nucleus with the ^{13}C neighbour should broaden the resonances as it was observed in the model compounds (Figure 3). Only A1 and A2 (Figure 4) have relatively small half-height line widths (0.5 ppm) similar to the carboxyl groups of the model compounds. Comparing these resonances with those of Table II, one can tentatively assign the two high-field signals at 170.2 (A1) and 171.2 ppm (A2) to protonated Asp which have to be localized within the hydrophobic core of the protein because under the experimental conditions chosen all external carboxyl groups are deprotonated (pH $\geq$6). The sharpness of the two high-field signals suggests that they are caused only by one group, respectively. Since the label in Glu/Gln is approximately half of the Asp/Asn label, only their cumulated signals could give rise to a signal as intense as in A1 or A2. It seems unlikely, therefore, that these resonances are due to more than one residue. However, it should be noted that the signals are more intense than, e.g., the C-13 ^{13}C resonance of retinal in bR (Harbison et al., 1985).

A further amino acid assignment can be made by considering the spectrum of bR^{blue} (Figure 5). In this sample, all hydrophilic, bulk water accessible carboxyl groups are protonated (Gerwert et al., 1987) and should absorb at the same position. Exactly this is observed in Figure 5 where a major band appears at 175 ppm. Thus, this band can be identified to represent protonated, water-exposed side chain carboxyl groups derived from aspartic acid. All signals downfield of 175 ppm should therefore be deprotonated hydrophilic carboxyls, whereas the signals below 175 ppm should arise from groups inside the membrane and/or membrane/water interface. The resonances which were assigned to protonated Asp (170.5 ppm, A1; 170.9 ppm, A2) are still present under these conditions, thus supporting the conclusions drawn above, namely, that they originate from internal protonated Asp. The small shoulders on the low-field side of the prominent signal might stem from residual deprotonated groups due to the acidity of the sample of around pH 4.5. Compared to native bR, the resonances A1 and A2 are associated with slightly different chemical shifts.

After the removal of the C-terminus containing Asp^{242}, it was expected either that one signal would disappear or that at least its intensity would decrease. Instead, two new relatively intense resonances at 178.0 ppm (C2) and 178.9 ppm (C3) appeared, and one signal at 177.2 ppm (C1) was enhanced. Their narrow bandwidth together with their chemical shifts is characteristic for deprotonated water-exposed carboxyl groups. The resonances do not disappear under conditions without cross-polarization. C1, C2, and C3 are under these conditions much more pronounced as the upfield carbonyl and the aliphatic signals (Figure 6, trace b), indicating that their relaxation time is less than 5 s. It is not understood why the bands also show up with strong intensity under conditions with cross-polarization (Figure 6, trace a).

The areas of the peaks C1, C2, and C3 are as already indicated under Results approximately proportional to the number of nuclei involved. The ratio of 1:2:1 for C1:C2:C3 therefore indicates that four aspartic acids are responsible for these signals.

The corresponding cation-depleted blue membrane of bR^{pap} has a MASS NMR spectrum quite similar to the native bR^{blue} (data not shown). This observation is in agreement with the conclusion that the carboxyl groups causing these signals are external aspartic acids. Since of the five existing external Asp,^{242}Asp together with the C-terminal tail was cleaved off by papain, the remaining four Asp are likely to correspond to residues located in the loops between helices AB and CD, respectively, both of which contain the sequence Asp-X-Asp (X = Pro/Ala). The appearance of resonances at higher fields and the concomitant depletion of absorbance between 174 and 176 ppm become plausible if one assumes that the environments of aspartate residues are changing from more saltlike conditions to an aqueous surrounding.

Apparently, the C-terminus folds over the membrane, thereby hiding certain parts of the protein. Such an immobilization of the C-terminus on the membrane surface was also deduced from fluorescence measurements with a fluorescent-labeled C-terminus (Renthal et al., 1983). However, Wallace and Henderson (1982) concluded from electron diffraction studies that the C-terminus is entirely disordered, allowing only the determination of an approximate location. Also, Marque et al. (1986) interpreted their fluorescence spectroscopic data to be consistent with the conclusion of Wallace and Henderson (1982).

Further support for the interaction of the C-terminal tail with the cytoplasmic membrane surface is given by sequence analysis of the cation binding site, the existence of which was proposed by several authors [see Ariki et al. (1987) and references cited therein]. In these experiments (Engelhard et al., unpublished results) two peptides, one from the C-terminal tail (including Glu^{232}-Ala-Glu) and the other from the loop region of helices CD (including Asp^{102}-Ala-Asp), were modified by a Co–pentaamino complex. Assuming that both peptides belong to the same binding site, it becomes understandable that upon removal of the C-terminus the Asp side chains are removed from a saltlike binding pocket and exposed to a more aqueous phase. If not the entire tail is fixed, at least the anchor of the C-terminus might be inflexible so that this interpretation is not contradictory to Marque et al. (1986), Wallace & Henderson (1982), and more recently Bowers and Oldfield (1988). These considerations impose some limitations on possible models of the bR topology. So far, it is not unequivocally established how the helices derived from the amino acid sequence are correlated to the electron density maps gained from neutron or X-ray diffraction studies [see, for example, Heyn et al. (1988)]. The above-described considerations placed the fragment Glu-Ala-Glu of the C-terminal

tail not too far from the loop of helices CD.

The assignments made in the above discussion ascribe two signals (A1 and A2) to protonated internal aspartic acids. IR experiments indicated that at least one protonated Asp is undergoing deprotonation/protonation changes during the photocycle (Engelhard et al., 1985). A minor band in the time-resolved difference spectrum was related to a protonated Glu residue. However, successive work (Eisenstein et al., 1987; F. Siebert, unpublished result) identified this signal as arising from an aspartic acid, leading to two interior protonated Asp. The NMR data clearly demonstrate that two Asp side chain carboxyl groups within the hydrophobic core of the protein are protonated. Therefore, of the four interior Asp residues, only two are deprotonated. To neutralize their negative charge, they require a counterion, one of which might be the protonated Schiff base.

Recent data from site-directed mutagenesis in which all internal Asp were replaced by either Asn, Glu, or Ala (Khorana, 1988; Mogi et al., 1988) were interpreted such that not one but two Asp residues, Asp^{85} and Asp^{212}, are interacting with the Schiff base. This was concluded from the observation that for each analogue a chromophore with an absorption maximum of $\lambda_{max} > 500$ nm was obtained, suggesting most of the specific protein–retinal interactions to be intact. This assignment leaves Asp^{96} and Asp^{115} as the protonated species. In IR studies of a mutant bR, Asp^{96} was identified as one of the protonated groups (K. Gerwert, B. Hess, J. Soppa, and D. Oesterhelt, personal communication). From structural models, it becomes evident that at least Asp^{85}, Asp^{96}, and Asp^{212} are in a position where a positive charge might be contributed by Arg^{82}, Arg^{75}, and the protonated Schiff base of Lys^{216}. So far, there is no chemical evidence for a protonated interior carboxyl group. However, the observation that Asp^{115} is modified by dicyclohexylcarbodiimide (Renthal et al., 1985), an agent known to react with protonated carboxyl groups, might be taken as an indication for such a possibility.

The assignment of the two unprotonated internal Asp of bR which were established by IR spectroscopy (Engelhard et al., 1985; Eisenstein et al., 1987) to resonances of the ^{13}C NMR spectra still remains unclear. In the spectrum of the papain-treated purple membrane, the four remaining external Asp and the two internal protonated Asp were clearly identified whereas the strong signal at 173 ppm (A3) could not be assigned to specific residues. It cannot be excluded, though, that this signal instead is due to the deprotonated carboxyl groups of two Asp. In this case, the Asn and Glu/Gln signals should actually be broader than expected and must be obscured by the wide underlying band. Future experiments on the M intermediate of bR should clarify this point. It should also be pointed out that the spectrum of bR^{blue} provides no evidence for protonation of an internal deprotonated Asp. A possible protonation of negatively charged groups was discussed to explain the purple–blue transition of the purple membrane on acidification or deionization [see, e.g., Kimura et al. (1984) and references cited therein].

Summarizing the results, it was shown that the hydrophobic interior of the protein contains two protonated aspartic acids which are probably participating in the light-triggered proton transfer. Five Asp residues have access to the bulk phase. Two of them might contribute ligands to a firmly bound intrinsic cation.

The incorporation of specifically labeled amino acids into bacteriorhodopsin gives the opportunity of studying their structural and functional properties. This method essentially offers a perturbation-free modification of an amino acid, allowing the analysis of the protein in its native state by infrared and solid-state NMR techniques.

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Registry No. L-Asp, 56-84-8; Ala-Asp, 20727-65-5; Gly-Asn-Ala, 119567-78-1; Gly-Gly-Asn-Ala, 104005-33-6; L-Trp, 73-22-3.

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