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Asp^{85} is the only internal aspartic acid that gets protonated in the M intermediate and the purple-to-blue transition of bacteriorhodopsin

A solid-state ^{13}C CP-MAS NMR investigation

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High-resolution solid-state ^{13}C NMR spectra of the ground state and M intermediate of the bacteriorhodopsin mutant $D_{96}N$ with the isotope label at [4-^{13}C]Asp and [11-^{13}C]Trp were recorded. The NMR spectra show that Asp^{85} is protonated in the M intermediate. The environment of Asp^{85} is quite hydrophobic. On the other hand, Asp^{212} remains deprotonated and a slight shift to lower field indicates a more hydrophilic environment. Asp^{85} also protonates in the purple-to-blue transition of bacteriorhodopsin in the deionized membrane, where it experiences a similar environment to M. The shift of Trp resonances in M reflect a conformational change of the protein in forming the M intermediate.

Bacteriorhodopsin; Blue bacteriorhodopsin; Solid-state nuclear magnetic resonance (NMR); Protonation change; Photocycle

1. INTRODUCTION

The retinal protein, bacteriorhodopsin (BR), forms a two-dimensional crystal lattice in the purple membrane (PM) of *Halobacterium halobium* and acts as a light-driven proton pump [1–3]. The protein spans the lipid bilayer in seven α-helices and the chromophore is covalently bound to Lys^{216} via a protonated Schiff base. A model for the structure of BR has been proposed using data from electron cryo-microscopy [4]. Two proton channels were deduced leading from the Schiff base to the cytoplasmic and the extracellular side of the membrane. The functional key residues in the extracellular channel include Asp^{85} and Asp^{212}, whereas Asp^{96} is located between the Schiff base and the cytoplasm.

After light absorption a reversible photocycle is initiated and results in a proton translocation across the membrane. Several intermediates can be distinguished by their visible absorption maxima and different models for the pumping mechanism were proposed [4–6]. The importance of M_{412}, the only intermediate of the photocycle with a deprotonated Schiff base, is common to all functional models. Its formation coincides with a fast proton release to the extracellular medium [7]. Several carboxyl groups are known to undergo protonation changes during the photocycle [8–10]. From infrared studies with BR mutants it was concluded that Asp^{85} and Asp^{212} become protonated in M_{412} [5]. The interaction of Asp^{212} with Tyr^{185} through a polarizable hydrogen bond which leads to the protonation of Asp^{212} in M was reported [11]. Contrary to these conclusions, Henderson et al. assume in their minimum model for proton pumping that Asp^{212} remains deprotonated throughout the photocycle [4]. Experiments have so far failed to unequivocally establish the role of Asp^{212}.

In addition to the function of Asp^{85} as proton acceptor in M, it has also been suggested that a carboxyl group near the Schiff base is involved in the purple-to-blue transition of bacteriorhodopsin [12–14]. The blue form, BR_{blue}, is obtained either by acid titration of removal of cations [15–18]. The protonation of Asp^{85} already in the blue BR ground state could be responsible for the lack of an M-like intermediate and the inability of this form to pump protons. On the other hand, FTIR investigations gave no evidence for protonation of an internal carboxyl group in blue BR [19] and thus spectroscopic confirmation for a protonated Asp^{85} is still needed.

On the basis of ^{13}C solid-state nuclear magnetic resonance (NMR) with [4-^{13}C]Asp/[11-^{13}C]Trp-labelled BR we were able to assign resonances to specific aspartic acids and determine the protonation state of the side chain carboxyl groups. Two protonated (Asp^{96}, Asp^{115}) and two deprotonated (Asp^{85}, Asp^{212}) aspartic acids were observed in the BR groundstate [20–22]. In this

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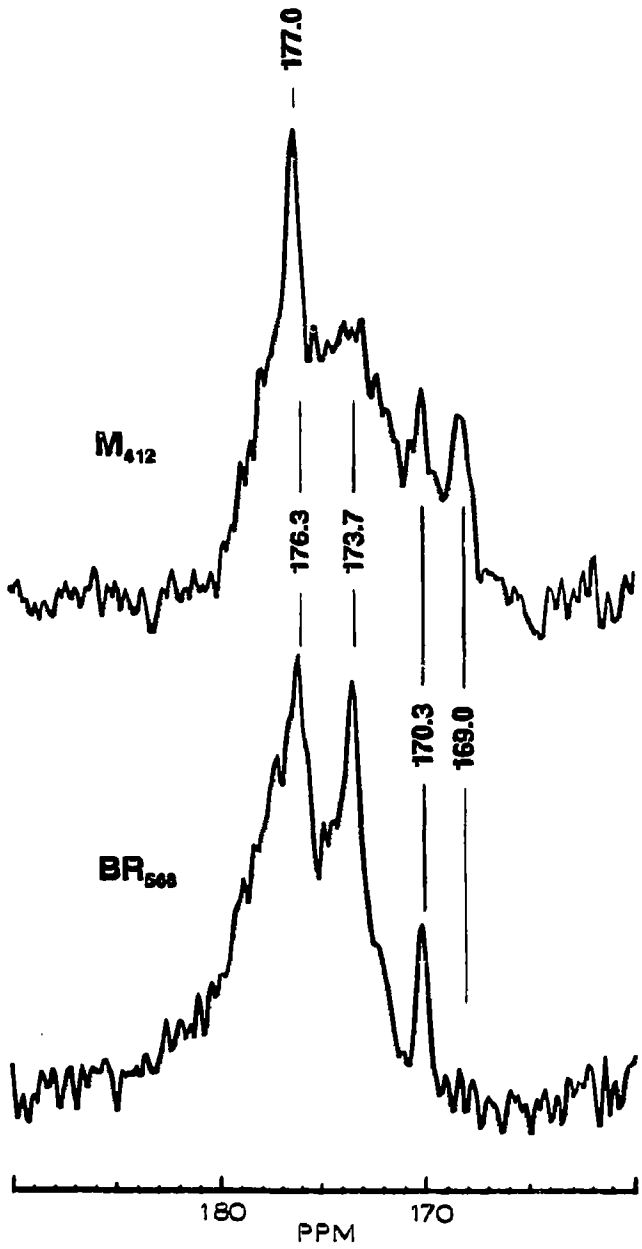

Fig. 1. ^{13}C CP-MAS difference spectra between $D_{96}N$ bacteriorhodopsin labelled with [4-^{13}C]Asp and native bacteriorhodopsin: ground state (bottom) and M intermediate (top).

study we have recorded spectra of the ground state and the M intermediate of the mutant $D_{96}N$ where Asp^{96} is replaced by Asn. In this mutant the decay of M is dramatically prolonged [23] allowing an easier stabilisation of the M intermediate under solid-state NMR conditions. The spectra reveal that only Asp^{85} (and not Asp^{212}) protonates in the M intermediate. Protonation of Asp^{85} could also be concluded from a spectrum of the blue form of wild-type BR. Conformational changes of the protein are reflected in changes of the Trp resonances both in the M intermediate as well as in blue BR.

2. MATERIALS AND METHODS

[4-^{13}C]Asp was incorporated into bacteriorhodopsin by growing the bacteria in a synthetic medium [10,20] and purple membrane was isolated following the standard procedures [24]. Wild-type bacteriorhodopsin was deionized by a cation-exchange reagent leading to the so-called blue BR which had an absorption maximum of 602 nm.

For the M_{412} studies a second sample was obtained from a mutant strain (PM 326) of *Halobacterium sp. GRB*, with a single site mutation in which Asp^{96} is replaced by Asn [23]. About 25 mg of lyophilized sample were transferred into a transparent sapphire rotor and 30 μl of borate buffer (0.5 M, pH 10) added. Rotating the sample for about 20 min with 2,000 Hz led to a thin layer of sample on the wall of the rotor. This allowed the illumination of the sample outside the magnet from all sides using a slide projector. After light adaptation the rotor was cooled to 220 K under constant illumination. The sample turned yellow, indicating the formation of M_{412}. The rotor was subsequently placed into the precooled NMR stator and the temperature was kept at 220 K throughout the whole experiment.

The spectra were recorded with the standard cross polarization (CP) sequence under magic angle spinning (MAS) on a Bruker CXP300 spectrometer (Bruker GmbH, Karlsruhe). The ^{13}C frequency was 75 MHz. We used a 3.5 μs 90° pulse time, a mixing period of 1.5 ms, a 50 ms acquisition time and a proton decoupling field equivalent to 75 kHz. The spinning speed was set at 3,250 Hz. A difference spectrum was obtained by subtracting the spectrum of native BR taken under identical conditions from the [4-^{13}C]Asp-labelled sample.

3. RESULTS AND DISCUSSION

The M-intermediate of the BR photocycle can be trapped by illumination at low temperature (220 K). In infra-red spectroscopy this fact is used to record BR–M difference spectra of thin film samples. The amount of PM necessary for NMR experiments (>20 mg) and the need to stabilize an intermediate for several hours in the inaccessible NMR magnet renders it rather difficult to

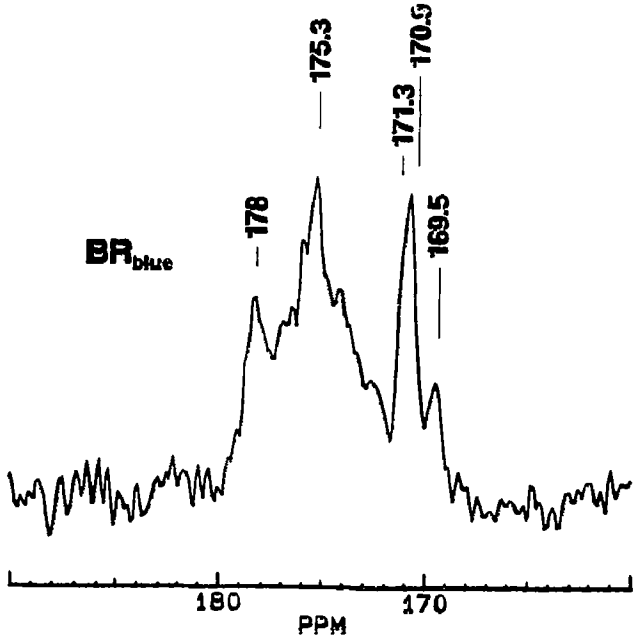

Fig. 2. ^{13}C CP-MAS difference spectra between bacteriorhodopsin labelled with [4-^{13}C]Asp and native bacteriorhodopsin: deionized form (BR_{blue}).

obtain NMR spectra of BR intermediates. Smith and co-workers [25] succeeded in forming M by illuminating the sample outside the rotor under a cold nitrogen gas flow and subsequently transferring the sample into the rotor at low temperature. They also had to use high pH and high salt concentrations or guanidinium-HCl to slow down the M decay. In this study we have used the BR mutant strain PM326 [23], which is known to have an M lifetime in the order of seconds at high pH [23]. This is due to the lack of Asp^{96} playing an important role in the reprotonation of the Schiff base. The mutant is spectroscopically identical to the wild-type and pumps protons across the membrane [23]. In addition, the mutant, $D_{96}N$, has the identical chromophore as wild-type BR both in the ground state and M [26]. In our earlier NMR studies [21,22] the spectra of the wild-type and the mutant, $D_{96}N$, did not differ as regards the internal Asp or the Trp resonances. Therefore it is reasonable to assume that the conclusions drawn for the residues Asp^{85} and Asp^{212} also hold true for wild-type BR.

Fig. 1 shows difference spectra of [4-^{13}C]Asp-labelled-minus native PM obtained from the light-adapted ground state and the M intermediate. The cross polarization technique favours the internal groups which are in a rather rigid environment [20]. Three pronounced signals are found in the ground state. They have formerly been assigned to the deprotonated Asp^{212} (176.3 ppm) and Asp^{85} (173.7 ppm) as well as to the protonated Asp^{115} (170.3 ppm) [20–22]. These assignments made use of FTIR studies [5] which showed that Asp^{115} is the second internal protonated aspartic acid in the BR ground state.

Before discussing the results, some remarks should be made on the effect of protonation regarding the ^{13}C NMR signal of a carboxyl group [27]. Regarding the chemical shift tensor of an aspartate as compared to a protonated carboxyl group, the most sensitive element is σ_{22} which experiences an upfield shift in the order of 20 ppm upon protonation. This dependency was also used to determine the protonation state of Asp residues in BR by evaluating the chemical shift anisotropy (CSA) from side band intensities in MAS spectra [22]. Protonation also leads to an upfield shift of the isotropic chemical shift (σ_{iso}). Therefore, comparing the σ_{iso} of identical chemical groups (as in the case of the internal Asp in the following conclusions) the protonation state can be characterized without knowledge of the full CSA tensor.

In the M spectrum the band near 173.7 ppm (Asp^{85}) has vanished and a new signal arises at 169 ppm. This shift clearly demonstrates that Asp^{85} takes up a proton during formation of M, confirming the results from FTIR [5]. The σ_{iso} is even higher than the resonance of protonated Asp^{115} at 170.3 ppm and thus argues for hydrophobic interactions of Asp^{85} in the M intermediate. A similar environment has already been proposed from FTIR investigations [10]. The resonance at 176.3 ppm (Asp^{212}) in the ground state shifts in M to a slightly lower field (177 ppm). Apparently Asp^{212} is still deprotonated with a minor change to a more hydrophilic surrounding. The signal of Asp^{115} at 170.3 ppm is affected in its intensity but no shift is observed. Thus it can be concluded that Asp^{115} remains protonated. Table I summarizes the results which support the pumping mechanism as described by Henderson [4]. It is clear that Asp^{85} is the acceptor for the Schiff base proton. However, Asp^{212} remains definitely deprotonated in M. Thus it appears unlikely that Asp^{212} is directly involved in the reprotonation of the Schiff base in M.

The difference spectrum between labelled and native BR in the deionized membrane (Fig. 2) shows a signal near 169.5 ppm which is not found in the purple form. The strongest signal at 173 ppm (Asp^{85}) in purple membrane is missing in the deionized form. Again this can be explained by a protonation of Asp^{85} resulting in a new band at 169.5 ppm with an almost identical σ_{iso} as that found in the M intermediate. The signals of Asp^{96} and Asp^{115} now merge into one broad band due to the shift of the resonance of Asp^{115} from 170.3 to 171 ppm, as previously shown [20]. Both residues are still protonated. The blue BR spectrum shows a strong band near 175.3 ppm, and a weak signal near 178 ppm becomes visible. The internal deprotonated Asp^{212} is considered to contribute to the signal at 175.3 ppm, having a slightly different environment going from the purple to the blue form. An additional contribution of external protonated Asp, as stated earlier [20], is likely. A still deprotonated external Asp may give rise to the signal near 178 ppm*.

*These observations can be seen in our earlier publication [20]. The peak near 169 ppm was barely visible as a slight shoulder and no attention was paid to it at that early stage of our studies.

Table I

Protonation state of internal aspartic acids and their isotopic chemical shifts

Residue	$D_{96}N$ mutant, ground state		$D_{96}N$ mutant, M_{412}		Wild-type, blue BR	
Asp^{85}	COO^-	173.7	COOH	169.0	COOH	169.5
Asp^{96}	–	–	–	–	COOH	171.3
Asp^{115}	COOH	170.3	COOH	170.3	COOH	170.9
Asp^{212}	COO^-	176.3	COO^-	177.0	COO^-	175.3

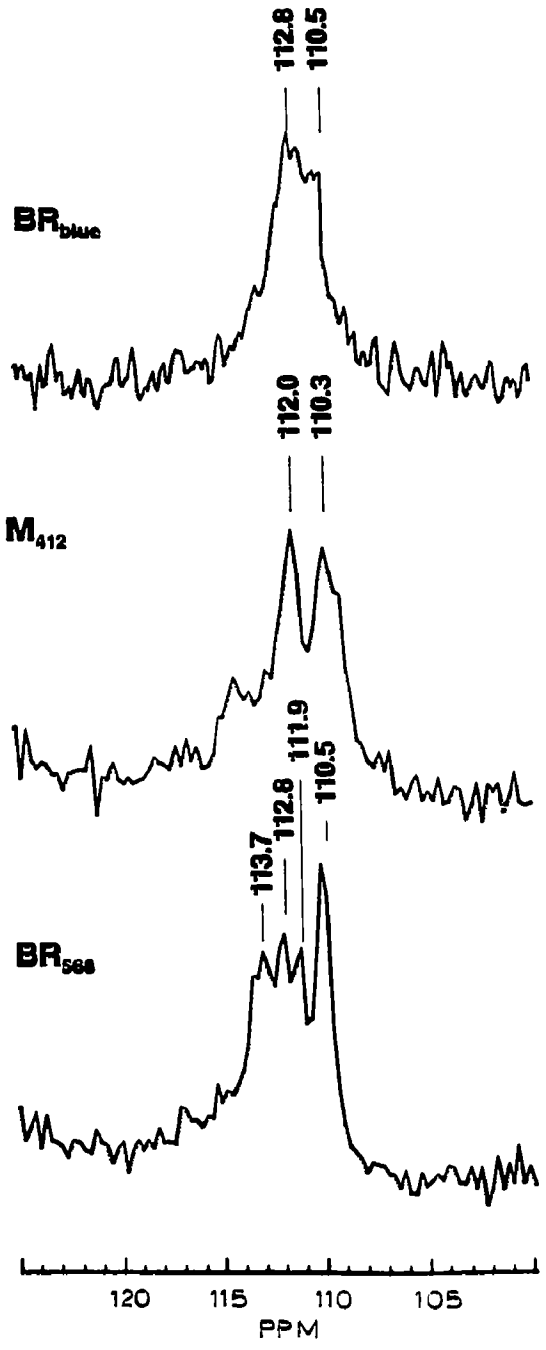

Fig. 3. ^{13}C CP-MAS spectra of $D_{96}N$ bacteriorhodopsin labelled with [11-^{13}C]Trp: BR ground state (bottom), M intermediate (middle) and deionized form (BR_{blue}).

Growing the bacteria on a synthetic medium containing [4-^{13}C]Asp also results in a high amount of ^{13}C in the C11 position of Trp residues [20]. Fig. 3 shows the aromatic region of the spectra of the $D_{96}N$ ground state, M_{412} and wild-type blue BR. Changes between the spectra of the ground state and M can be discerned. A decrease in intensity of the strongest band at 110 pm and near 113 pm is found in the M spectrum which now shows only two broad signals. The signals are even more influenced in the deionized membrane.

Although no assignment to specific amino acids is possible, some conclusions can be drawn. From ^{13}C NMR relaxation time experiments it was concluded that the signals at 113.7 and 110.5 ppm belong to internal Trp in a rigid environment leading to a relaxation behaviour similar to that of the internal Asp [22]. Therefore internal Trp residues close to the chromophore [4] are affected by retinal isomerization upon formation of M. Such perturbations of Trp in M were also reported for UV and FTIR difference spectra [28,29]. In the case of the blue membrane the severe perturbation of the Trp resonances argues for larger structural alterations.

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