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# High resolution $^{13}$C-solid state NMR of bacteriorhodopsin: assignment of specific aspartic acids and structural implications of single site mutations *

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**Abstract.** Three mutant strains of *Halobacterium sp. GRB* with the site of mutation in the bacterioopsin gene (PM 326: Asp96 → Asn; PM 374: Asp96 → Gly; PM 384: Asp85 → Glu) were grown in a synthetic medium containing (4-$^{13}$C)-Asp. The mutant bacteriorhodopsins labeled with (4-$^{13}$C)-Asp (37%–45%), and owing to the metabolism of *Halobacteria* also with (11-$^{13}$C)-Trp (50%–100%), were isolated as purple membranes and $^{13}$C Solid State Magic Angle Sample Spinning (MASS) Nuclear Magnetic Resonance (NMR) spectra of the samples were taken. The Asp96 mutants lacked the signal at 171.3 ppm which was previously assigned to a protonated internal Asp (Engelhard et al. 1989a). This observation supports the conclusion that Asp96 is protonated in the ground state. PM 384 (Asp85 → Glu) has an absorption maximum at 610 nm. It can be converted into a purple form ($\lambda_{max} = 540$ nm) by treatment with a detergent (CHAPSO). The NMR-spectra of these two species differ from each other and from the wild type. The intensity of the resonance at 173 ppm in the wild type spectrum is reduced in both forms of the mutant protein. It is probable that this signal is caused by Asp85. The amino acid changes result not only in a perturbation of their direct environment but also effects on Trp residues and the chromophore protein interaction can be observed.

**Key words:** Bacteriorhodopsin – Solid state NMR – Stable isotope labeling – Perturbation free analysis of mutants – Assignment of specific aspartic acids

The vectorial proton transfer in bacteriorhodopsin (bR), the light driven proton pump from *Halobacterium halobium*, is thought to be actuated by the isomerization of the retinal chromophore (for recent reviews on bacteriorhodopsin see Stoeckenius and Bogomolni 1982; Ovchinnikov et al. 1982; Lanyi 1984; Oesterhelt and Tittor 1989). Early models of the mechanism of the proton pump considered mostly the protonation- and isomerization state of the retinal Schiff base (e.g. Schulten and Tavan 1978; Lewis 1979). In these concepts the retinal functioned as a switch between the ejection and the injection pathway forming a proton donating/accepting complex within the protein (Fischer and Oesterhelt 1979; Fischer and Oesterhelt 1980). The ensuing steps of the proton transfer were described in more general terms using networks of hydrogen bonds (e.g. Nagle and Tristam-Nagle 1983). Also, similar mechanisms were proposed which relied on specific amino acid residues (e.g. Merz and Zundel 1981).

Experimental evidence for the participation of specific amino acid residues in the reaction cycle of bR was first provided by the analysis of UV absorption changes (Kuschmitz and Hess 1982; Hanamoto et al. 1984; Ovchinnikov et al. 1986) and was attributed to Tyr residues. This could be confirmed by FTIR difference spectroscopy (Dollinger et al. 1986; Roepe et al. 1987) on native and mutant bR which proved that the effects were confined to Tyr185 (Braiman et al. 1988a).

Infrared experiments demonstrated also that carboxyl groups ascribed to Glu and/or Asp residues are protonated and deprotonated during the photocycle (Rothschild et al. 1981; Siebert et al. 1982). Structural models of bR (Trewhella et al. 1983) placed four Asp residues inside the hydrophobic core of the protein and it was assumed that these amino acids should be part of the proton transfer chain. In further investigations using isotopically labeled bR it was confirmed that four Asp residues from the hydrophobic interior of the protein are involved in the reaction cycle (Engelhard et al. 1985; Eisenstein et al. 1987). These four Asp residues were recently replaced in mutant proteins generated by site directed mutagenesis (Mogi et al. 1988) as well as in phototrophically negative mutants (Soppa and Oesterhelt

*Abbreviations*: CHAPSO, 3-(3-cholamidopropyl)-dimethylammonio-2-hydroxy-1-propane sulfonate; CP, crosspolarization; bR, bacteriorhodopsin; FTIR, Fourier-transform-infrared; FID, free induction decay; IR, Infrared; MASS, Magic angle sample spinning; NMR, Nuclear magnetic resonance; TMS, tetramethylsilane

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1989) by Glu and/or Asn. It could be demonstrated that these mutations affect the chromophore and/or proton pump efficiency (Marinetti et al. 1989; Holz et al. 1989; Butt et al. 1989). FTIR difference spectroscopy could specifically identify the bands of the side chain carboxyl groups from Asp85, Asp96, Asp115 and Asp212 (Braiman et al. 1988 b; Gerwert et al. 1989). From these experiments it could be deduced that Asp96 and Asp115 are protonated in the ground state.

Two internal protonated Asp residues were also determined by solid state NMR measurements of $^{13}$C-labeled bR (Engelhard et al. 1989 a). These data provided the evidence for the existence of four classes of Asp residues which can be distinguished by their chemical environment. Of the nine Asp contained in bR five residues are located on the surface of the membrane protein. At least two of them experience an ionic environment which is altered by removing the C-terminal tail. Also, two protonated internal Asp could be discerned. However, two internal deprotonated Asp could only be tentatively correlated with a signal at 173 ppm.

An unequivocal assignment of the resonances to specific amino acid residues can be accomplished by chemical or genetic modifications of single amino acids. This would also allow the characterization of the chemical environment of specific groups within the protein. Furthermore, the effects of defined perturbations of the protein on the function of these residues can be studied in detail.

Recently, the bR opsin gene of *Halobacterium sp. GRB* was isolated and sequenced. The derived primary structure of the protein was found to be identical to that of *Halobacterium halobium*. Mutagenesis of *Halobacterium sp. GRB* and selection of phototrophically negative strains led to the characterization of mutant bR's in their natural environment, that is the purple membrane (Soppa and Oesterhelt 1989; Soppa et al. 1989). Among these were mutants in which Asp96 was replaced by Asn or Gly and Asp85 is changed to Glu. In the experiments described here these latter mutant strains were grown on a synthetic medium containing (4-$^{13}$C)-Asp. Thus, the corresponding $^{13}$C-labeled bR's were isolated and analysed by solid state $^{13}$C NMR measurements.

## Materials and methods

### *Materials*

All chemicals used were reagent grade. D,L-(4-$^{13}$C)-Asp was purchased from Isotec, Miamisburg, Ohio. The $^{13}$C-content and position of the label were analysed by Mass Spectrometry prior to use. One liter of basal salt solution was composed of 250 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, and 3 g Na-citrate in $H_2O$. $^{13}$C-Asp labeled wild type bacteriorhodopsin was obtained from *Halobacterium halobium* (strain $R_1M_1$) as described by Engelhard et al. (1985).

### *Growth of bacteria*

The mutant strains were obtained from *Halobacterium sp. GRB* as described by Soppa and Oesterhelt (1989). The mutant strain 326 contained Asn and strain 374 contained Gly instead of Asp96. In strain 384, Asp85 was replaced by Glu.

The mutants were grown in a synthetic medium according to Engelhard et al. (1985) in which Asp was replaced by (4-$^{13}$C)-Asp and (4-$^{14}$C)-Asp.

### *Isolation of mutant bR*

The bacteria were harvested in the late logarithmic phase by centrifugation and washed three times with 3 M basal salt solution (solution A). After the last centrifugation 1 mg DNase in 5 ml solution A was added to the pellet. The cells were disrupted by three freeze ($-70\,^\circ$C) – thaw (20 °C) cycles. After the last thawing the sample was kept at room temperature overnight. The resulting mixture was centrifuged at 45,000 g and washed once with solution A. The final pellet was resuspended in solution A and centrifuged for 5 min at 500 g to remove most of the cell debris. The bR-containing supernatant was collected and the sediment was washed at the same centrifugal force until an almost white pellet was obtained. The combined supernatants were sedimented at 45,000 g and the pellet was washed 4 times with solution A and subsequently 5 times with distilled water. The final pellet was further purified using a sucrose density gradient as described by Oesterhelt and Stoeckenius (1974). The fractions containing bR were washed with distilled water to remove the sugar. The yield from 1 l of culture was 6.3 mg Asp85 → Glu from PM 384, 30 mg Asp96 → Asn from PM 326, and 23 mg Asp96 → Gly from PM 374.

The purity of the mutant bR preparations was determined by their absorption spectra, their infrared spectra, and by SDS-Gel electrophoresis. The ratio between the absorption maximum of the chromophore and the protein was 1 : 1.8 for native bR. The values for the mutant bR were 1 : 1.9 for PM 384, 1 : 1.8 for PM 326 and 1 : 1.8 for PM 374. SDS-Gel-electrophoresis revealed that the mutants co-migrated with native bR establishing that the molecular weights were similar to the wild type and that the C-terminus was not cleaved off during the purification procedure.

When PM 384 was treated with 0.8% (w/v) CHAPSO the absorption maximum shifted from 610 nm to 540 nm with a shoulder at 620 nm indicating that not all of the 610 nm species was converted to the 540 nm compound. The sample was sedimented and the pellet was lyophilized.

### *Determination of the $^{13}$C-enrichment*

The isotope enrichment in Asp was determined radioactively by analyzing the incorporation yield of (4-$^{14}$C)-Asp as described by Engelhard et al. (1985) (PM 374: 44%; PM 326: 37%; PM 384: 70%). In the case of

PM 326 and PM 384 the incorporation was also confirmed by infrared difference spectroscopy by comparing the area of the $^{12}$COOH- and $^{13}$COOH-stretching vibrations of Asp residues in the bR-M difference spectrum (Engelhard et al. 1985).

The insertion of the isotope into the C-11 position of Trp (Engelhard et al. 1989a) was also observed in the mutants and the amount was similar to the wild type as determined by a comparison of the corresponding $^{13}$C NMR signals of Trp and the aliphatic carbons.

### $^{13}$C-NMR measurements

For the NMR-measurements purple membrane in unbuffered $H_2O$ was lyophilized and transferred to the rotor. Subsequently, the samples were rehydrated with an equal amount of distilled water (weight/weight). Before lyophilisation, the samples had a pH of 6.

All $^{13}$C-NMR-spectra were obtained from approximately 60 mg purple membrane samples except for PM 384 where less than 15 mg purple membrane were available.

Measurements were performed on a Bruker CXP 300 NMR Spectrometer (Bruker GmbH, Karlsruhe) operating at 75 MHz for $^{13}$C. All spectra were taken at room temperature with spinning speeds of 3,250 Hz. All shifts are given relative to external TMS.

Spectra were acquired with and without the standard cross polarisation (CP) technique under proton decoupling with a corresponding frequency of 75 kHz. The FID's were detected for 50 ms and typically $2-3 \times 10^4$ scans were accumulated. The 2 K data points obtained were zero-filled up to 4 K, processed by a 10 Hz line broadening and Fourier-transformed. In all cases the recycle delay was 5 s and for CP spectra the mixing time under adjusted Hartmann-Hahn conditions was 1.5 ms (for a monograph on Solid State NMR see Mehring (1983).

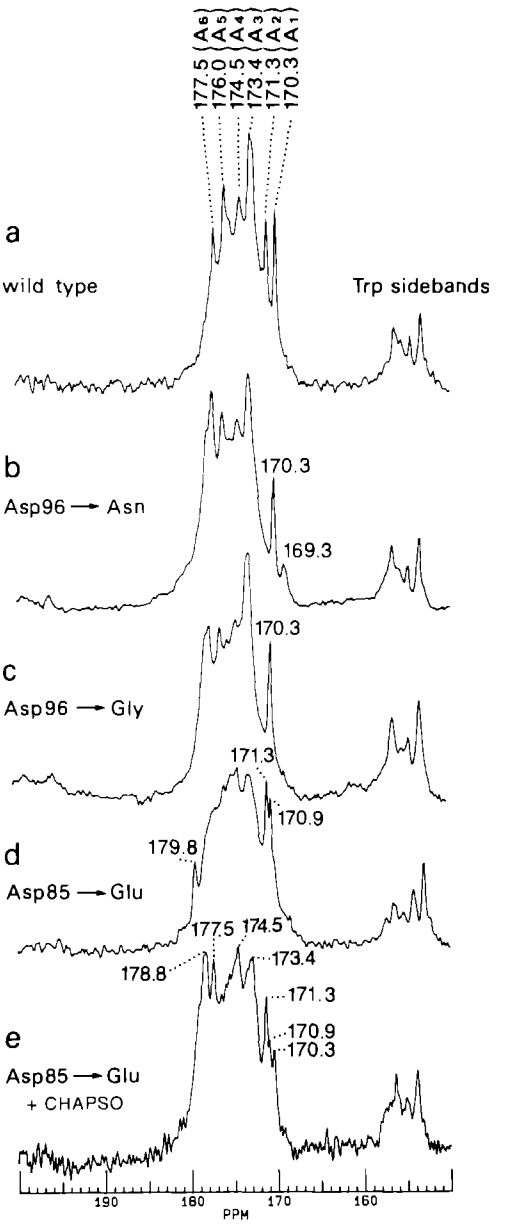


**Fig. 1a–e.** $^{13}$C-Solid-State NMR spectra of the carbonyl region of (4-$^{13}$C)-Asp labeled bRs. The spectra were taken with cross-polarization. **a** wild type bR **b** Asp96→Asn (PM 326) **c** Asp96→Gly (PM 374) **d** Asp85→Glu (PM 384) and **e** Asp85→Glu+CHAPSO

## Results

### Carbonyl-resonances

The $^{13}$C-NMR-spectral area of the carbonyl-resonances is shown in Fig. 1. In the wild type (Fig. 1a) six signals (A1–A6) appear which were attributed to protonated internal Asp residues (A1, A2), to deprotonated internal Asp carboxyl groups and/or Asn residues (A3) and to external carboxyl groups (A4, A5, A6) (Engelhard et al. 1989a).

*Asp96→Asn mutant:* Comparing the carbonyl region of the wild type (Fig. 1a) with the Asp96→Asn mutant (Fig. 1b) the band at 171.3 ppm (A2) is missing in the mutant bR indicating that A2 is due to Asp96. A new signal with a half-width greater than that of A2 and a much lower intensity is observed at 169.3 ppm. This resonance can be assigned to Asn96.

The low field region of the mutant spectrum is also not identical with that of the wild type. The most pronounced difference is an increase in intensity around 178 ppm. This is even more obvious in the spectrum which was taken without cross-polarization (Fig. 2a, b), where a new broad band arises at 177.8 ppm. Additionally, the sharp peaks at 175.2, 174 and 173.2 ppm are not observed in the corresponding wild type spectrum.

Similar spectral features are also seen in the spectra of the other mutant bR species (see below) and might be correlated to the site of mutation. However, the spectra of the wild type were taken from samples isolated from strain $R_1M_1$. Therefore, we cannot exclude the possibility that the observed differences are due to an altered surface structure mediated, for example, by a different

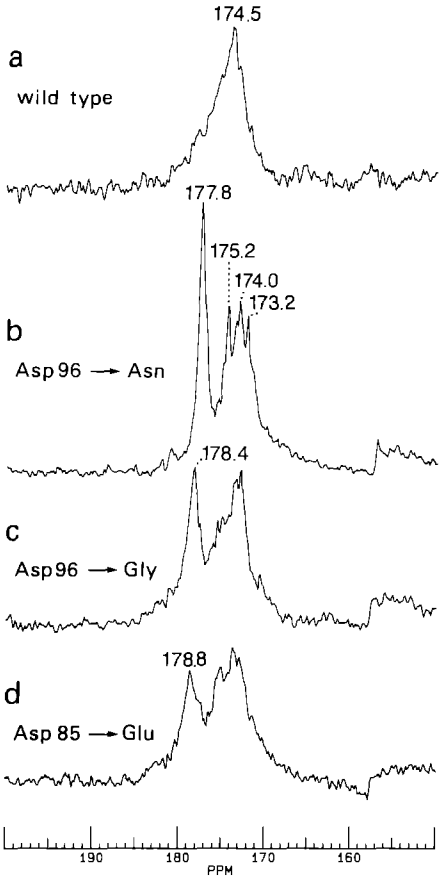


**Fig. 2a–d.** $^{13}$C-Solid-State NMR spectra of the carbonyl region of (4-$^{13}$C)-Asp labeled bRs. The spectra were taken without cross-polarization. **a** wild type bR **b** Asp96 → Asn (PM 326) **c** Asp96 → Gly (PM 384) **d** Asp85 → Glu (PM 384)

lipid composition. Indeed, preliminary experiments using (4-$^{13}$C)-Asp bR from *Halobacterium sp. GRB* showed similar effects.

*Asp96 → Gly mutant:* As with the spectrum of the mutant Asp96 → Asn the signal at 171.3 ppm (A2) is missing in the spectrum of the mutant Asp96 → Gly (Fig. 1c, 2c). This mutation removes the carboxyl group of Asp96 but does not introduce a new carbonyl group like that of Asn96. Consequently, the resonance at 169.3 ppm is not observed supporting the above conclusion. The relative intensities downfield of 174 ppm are altered as compared to the wild type with an increase again around 178 ppm. The non-CP spectrum (Fig. 2c) shows a new peak at 178.4 ppm with a half-width of 2 ppm but a smaller intensity as compared to the Asp96 → Asn mutant. The peak at 170.3 ppm is barely visible without CP.

*Asp85 → Glu mutant:* The CP-spectrum of this mutant in its natural lipid environment differs considerably from all other samples (Fig. 1d, 2d). The band at 170.3 ppm is slightly shifted to 170.9 ppm. Relative to this signal and to A2 at 171.3 ppm, the intensity of A3 is reduced. The pattern of the signals between 174 and 179 ppm of the wild type disappeared and only a broad band with a maximum at around 175 ppm composed of overlapping resonances remains, but a new sharp resonance is observed at 179.8 ppm.

In the non-CP spectra a broad band similar to the other mutants arises around 178.8 ppm with an intensity less than that of the Asp96 → Asn mutant but comparable to that of Asp96 → Gly.

The spectrum of the CHAPSO modified PM 384 again exhibits a well resolved fine structure (Fig. 1e). The low-field peak at 179.8 ppm has disappeared and bands at 178.8 and 175.5 are clearly visible. The high-field line is again located at 170.3 ppm as in the other spectra. However, a small band at 170.9 ppm is still discernable. This signal is probably due to the remaining 610 nm species. In both Asp85 → Glu spectra the broad feature at 174.5 ppm is present and the band at 173.4 ppm is reduced. An additional spectrum without cross polarization could not be taken because only 15 mg of this mutant were available.

### *Trp-resonances*

The sharp resonances of the wild type between 110 and 115 ppm which can be attributed to the C-11 position of Trp residues are not detected without cross-polarization and vary only slightly in the Asp96 mutants (Fig. 3).

However, the Asp85 → Glu mutant differs in this spectral range (Fig. 3d). The band at 113.8 ppm of the wild type spectrum is decreased and the signal at 112.8 ppm is shifted to 113.3 ppm. After treating this sample with CHAPSO (Fig. 3e) the band at 113.8 ppm shifts, probably to 113.3 ppm, and therefore increases its intensity.

## Discussion

Ample information exists about the distribution and protonation state of Asp residues in bR (Engelhard et al. 1985; Eisenstein et al. 1987; Mogi et al. 1988; and Engelhard et al. 1989a). Five deprotonated Asp are located on the cytoplasmic surface of the membrane. Two of the four internal Asp are protonated. The other two Asp are deprotonated, thus requiring an environment which is able to neutralize their charge. By analyzing the $^{13}$C-NMR spectrum of the mutant bR and comparing it with the spectrum of the wild type the signals can be assigned to specific aspartic acids. Furthermore, the distribution of the isotope allows one to evaluate the effects of the mutations on other parts of the protein.

### *Assignment of resonances*

In the solid state NMR spectrum of the two mutants Asp96 → Asn and Asp96 → Gly the signal at 171.3 ppm (A2) which was present in the spectrum of the wild type is clearly missing, thus indicating that this resonance can be assigned to Asp96. In earlier experiments (Engelhard et al. 1989a) this peak was attributed to an internal protonated aspartic acid. From these data it follows that in dark-adapted bacteriorhodopsin Asp96 is protonated. This result provides independent evidence for the same

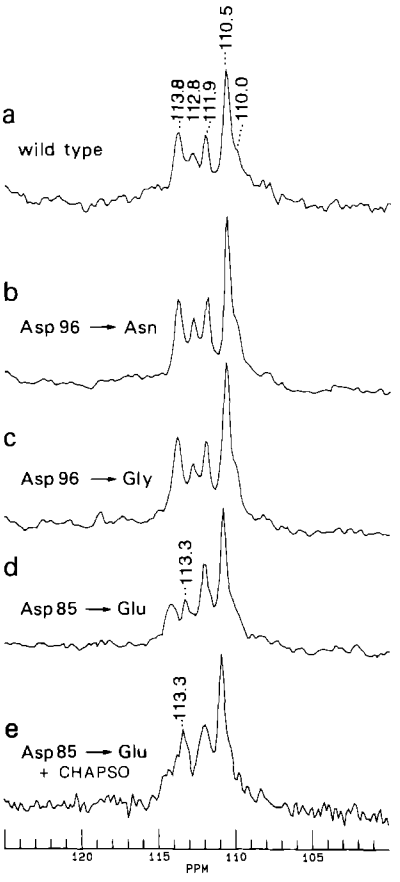


**Fig. 3a–e.** $^{13}$C-Solid-State NMR spectra of the C-11 Trp region of **a** wild type bR **b** Asp96→Asn (PM 326) **c** Asp96→Gly (PM 374) **d** Asp85→Glu (PM 384) and **e** Asp85→Glu treated with CHAPSO. The spectra were taken with cross-polarization

conclusion drawn from FTIR difference spectroscopy of the same mutant (Gerwert et al. 1989) and of mutants obtained by site directed mutagenesis (Braiman et al. 1988 b).

The second sharp resonance at 170.3 (A1) also generated by a protonated carboxyl group is not altered by the mutation. Braiman et al. (1988 b) conclude from their IR data that in the ground state the second protonated Asp is Asp115. This suggests that Asp115 gives rise to A1, confirming earlier conclusions (Engelhard et al. 1989 a).

The new small peak in the spectrum of the Asp96 →Asn mutant at 169.3 ppm can be assigned to the Asn96 residue. The intensity is much smaller and the half-height width larger than that of the protonated carboxyl groups. This is to be expected since polypeptide model compounds already revealed this difference between Asp and Asn carbonyl resonances (Engelhard et al. 1989 a). The broader half-width can be explained by the remaining dipolar coupling to the quadrupole moment of the adjacent $^{14}$N which will not be totally removed by magic angle spinning (Hexem et al. 1981).

There are three Asn in bacteriorhodopsin which must have resonances in the range between 169 and 179 ppm. As stated earlier, the bands downfield from 175 ppm are attributable to deprotonated external Asp (Engelhard et al. 1989 a). It still remains uncertain whether the broad intense peak at 173.4 ppm is due to internally deprotonated Asp and/or Asn. But taking the intensity of the single Asn peak at 169.3 ppm into consideration, it is more likely that this major peak reflects the remaining two internal Asp. Probably the Asn resonances can only be observed if they are not obscured by other strong resonances.

It should be noted that the amide group of Asn96 is better shielded than all other carbonyl groups. One might conclude that the Asn residue is in a more hydrophobic chemical environment than the other naturally occuring Asn residues.

As already mentioned, IR experiments provided evidence for two deprotonated and two protonated internal Asp. Since Asp96 and Asp115 are protonated it can be concluded that Asp85 must be an internal, deprotonated Asp. Having assigned Asp115 to A1 and Asp96 to A2 the question arises which resonance(s) of the spectrum originate(s) from the two internal deprotonated Asp. A peak arising from Asp85 should vanish upon mutation of this group as in the case of the band at 171.3 ppm of the Asp96 mutants. Comparing A3 in all four samples with the peaks of the protonated Asp the relative intensity of the signal at 173.4 ppm is smaller in the untreated as well as in the CHAPSO-treated Asp85 → Glu mutant protein. It is therefore likely that this signal is due to Asp 85. However, it should be emphasized that the dramatic spectral changes seen in Asp85 → Gly do not allow an unequivocal assignment such as in the case of Asp96 →Asp.

The Asp85 → Glu mutant has an absorption maximum of 610 nm. A shoulder at 540 nm indicates that this bR-mutant is in equilibrium with another state. The corresponding mutant obtained by site-specific mutagenesis and reconstituted into vesicles possesses the normal absorption maximum of a reconstituted purple membrane at 560 nm (Mogi et al. 1988). The different absorption maxima indicate that the chromophore responds to distinct environments and this variability is connected to the mutation at position 85. It should be noted that only the 540 nm species functions as an active proton pump as was shown by the action spectrum obtained by photocurrent measurements (Butt et al. 1989). This observation finds an analogy in the NMR spectra of the 610 nm and 540 nm species of PM 384 where only the spectrum of the purple form resembles roughly the native configuration. The steric and conformational interplay of the protein groups is quite sensitive to this site of mutation and, interestingly, the effects can be partly reversed by the external modification of the lipid bilayer.

The origin of the sharp new resonance at 179.8 ppm remains unidentified. It seems most likely that an external Asp altered its environment substantially, thereby causing a shift to lower fields, which is reversed in the CHAPSO-treated mutant.

### *Structural implications of the mutations*

The chemical modification of proteins has been widely used for the elucidation of the relationship between biological properties and specific amino acid residues. However, such studies often failed to be successful because it

was impossible to determine the exact nature of the chemical modification. Even in cases where the site(s) was (were) known the observed effects could not be unequivocally ascribed to the modified amino acid(s) alone. The recent development of site-directed mutagenesis provided an elegant way to replace an amino acid by another one at a distinct position in a protein. However, the basic problem of chemical modification still remains. Thus, it is unclear whether the altered functional properties of the mutant-derived protein are caused by the site of mutation itself, or whether the mutation is only indirectly associated with the catalytic site. However, structural analysis by crystallography, high resolution two and three dimensional NMR, and solid state NMR offer the possibility of answering these questions (e.g. Alber et al. 1987, 1988, and literature therein).

The $^{13}C$ isotope distribution in bR provides an internal antenna for structural changes at different positions of the protein. Generally, there are five groups which can be monitored. The external Asp indicate changes on the membrane surface, the internal protonated Asp96 and Asp115 and the deprotonated Asp85 might give data on the proton transfer chain, and C-11 of the Trp aromatic ring registers changes within the hydrophobic core of the protein. The following effects of the mutations on these sites of bR become evaluable.

The change of relative signal intensities between 173 and 179 ppm (Fig. 1) might indicate that a modification of a single residue embedded in the interior of the protein causes environmental changes for groups on the external sides of bacteriorhodopsin. In the non-CP spectra with short recycle delay (5 s) where one would expect to detect the mobile groups this substantial alteration of the spectra becomes particularly evident (Fig. 2). However, it is important to note that these intensity changes might not be due to the mutations as such but to differences of external parameters which originated from the particular strain. To understand these observations further investigations are necessary.

As already mentioned, no pronounced variations in the Trp resonances were observed with the exception of the Asp85 → Glu mutant where effects of the low-field Trp-resonances occurred. This is of special interest because four out of eight Trp residues are thought to sandwich the retinal chromophore (Polland et al. 1986). In the 540 nm and 610 nm form of PM 384 one band seems to be mostly affected, indicating that the corresponding Trp residue(s) might be positioned close to the site of mutation.

Another example of perturbations at the site of Trp residues is the cation-depleted bR, the so-called "blue membrane". After removal of cations from the purple membrane the substantial shift of the Trp resonances is indicative of conformational changes in the interior of the protein (Engelhard et al. 1989a). The effects in the Asp85 → Glu mutant are not as pronounced. This implies that here – in contrast to the blue membrane – the absorption change of the chromophore is not paralleled with greater internal conformational modifications of the protein itself. However, both in the blue membrane and in the blue mutant protein but not in Asp96 → Asn and Asp96 → Gly the peak at 170.3 ppm which was assigned to Asp115 is slightly shifted to lower fields. This suggests that in both samples Asp115 is located in similar chemical environments.

For a better understanding of the origin of these effects on Asp115 and the Trp resonances on the one hand and the lack of effects on the other hand, one should consider possible structural consequences of the mutations. On the basis of the analysis of crystal structures of various proteins it could be demonstrated that one polar side group of Asp residues has the ability to form up to four hydrogen bonds. In the interior of the protein it is likely that they will develop these bonds, leading to an extensive hydrogen-bonded network (Baker and Hubbard 1984).

The exchange of Asp by Asn, a conservative replacement, should not seriously influence the number of hydrogen bonds. Indeed, the thermal stability of the Asp96 → Asn is comparable to the wild type bR (Brouillette et al. (1989); for an analysis of the contributions of hydrogen bonds to the thermodynamic stability of proteins see, for example, Alber et al. (1988), and literature therein).

Contrary to these considerations, one would expect more severe perturbations from a change of Asp to Gly as in Asp96 → Gly. Gly – as opposed to Asn – is a poor helix-forming residue (Chou and Fasman 1978) and might therefore interfere with the helical alignment of helix C. Furthermore, since Gly has no side chain to form hydrogen bonds, this mutation disrupts the hydrogen bonded network of the Asp carboxylic group. Moreover, the resulting gap in the three-dimensional structure has to be filled by accomodations of the protein itself. It could also be possible that a water molecule occupies the vacancy as was observed, for example, in the case of a Thr157 → Gly mutant of phage T4 lysozyme (Alber et al. 1987).

It should be noted that both mutations slow down the M → bR transition similarly by more than one order of magnitude (Butt et al. 1989). This observation together with the almost identical small structural perturbations described above might support the conclusion that the hydrogen-bonded networks connecting the cytoplasmic surface and site 96 are similar in both mutants. The retardation of the reprotonation of the Schiff-base might be explained either by the functional insufficiency of the hydrogen-bonded network or by the necessity to deprotonate Asp96 in the M → N transition. The latter possibility was suggested from FTIR-measurements (Gerwert et al. 1989).

The substitution of Asp by Glu as in the case of PM 384 (Asp85 → Glu) does not involve a change in the negative charge, however, it requires more space to accomodate the additional methylene group. The thermodynamic stability of this mutant is comparable to the wild type (Brouillette et al. 1989) indicating a similar hydrogen-bonding potential in both proteins. Apparently, the protein undergoes conformational changes to compensate for the additional group and one might speculate that this site belongs to a more rigid part of bR. However, one should consider that the effects observed in

Asp85 → Glu might also be due to a protonated carboxyl group of glutamic acid.

As detailed above specific mutations in bR have structural effects on other parts of the protein. These perturbations become discernable by the spectroscopic behaviour of specific groups of the protein. Some of these groups are of functional importance, such as Asp85 and Asp96, whereas others may interact with the retinal chromophore, such as Trp and Asp115.

These observations have some implications for the interpretation of functional data gained from modified proteins. The altered function is not necessarily due only to the mutated site. To distinguish between direct and indirect effects the analysis of the structure is desirable if not mandatory.

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