Aspartic Acid-212 of Bacteriorhodopsin Is Ionized in the M and N Photocycle Intermediates: An FTIR Study on Specifically ^{13}C-Labeled Reconstituted Purple Membranes†

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Received December 29, 1992; Revised Manuscript Received March 16, 1993

ABSTRACT: Purple membrane was regenerated from the denatured proteolytic (protease V8) fragments V-1 and V-2 of bacteriorhodopsin (BR), native membrane lipids, and *all-trans*-retinal. FTIR difference spectra of M and N intermediates of the reconstituted system are in close correspondence to those obtained from native BR. Asp-212 is the only internal aspartic acid in the V-2 fragment (helices F and G). Reconstituting a V-2 fragment from a [4-^{13}C]Asp-labeled BR preparation with an unmodified V-1 fragment and vice versa have allowed us to assign IR bands to either Asp-212 or any of the remaining aspartic acids on V-1 (helices A–E). A carboxylate vibration at 1392 cm^{-1} has been identified in the M and N intermediates and assigned to Asp-212. Since no contribution of this residue to C=O stretches of protonated carboxyl groups was detected, Asp-212 must be ionized in light-adapted BR as well. The effect of [4-^{13}C]Asp labeling of V-1 revealed a carboxylate vibration at 1385 cm^{-1} in light-adapted BR. Since Asp-96 and Asp-115 are protonated, this band is caused by Asp-85. All absorption changes of C=O stretches of protonated carboxyl groups are due to Asp residues on V-1. Correspondingly, the proton acceptor for Schiff base deprotonation in M is located on V-1, and must be Asp-85 (the only ionized Asp on V-1). The band assignments are compared with those reported for BR mutants, and the potential role of Asp-212 for proton translocation is discussed.

Bacteriorhodopsin (BR)[1] is a membrane protein of *Halobacterium halobium* which enables the archaebacterium to survive photosynthetically under lack of oxygen [for reviews, see Stoeckenius et al. (1979), Stoeckenius and Bogomolni (1982), Khorana (1988), and Oesterhelt and Tittor (1989)]. BR is composed of seven transmembrane α-helices. It is found within the cell membrane in densely packed patches of two-dimensional crystalline order, i.e., the purple membrane (PM). Light is absorbed by the chromophore *all-trans*-retinal, which is covalently linked to Lys-216 of the apoprotein via a protonated Schiff base (Rothschild & Marrero, 1982; Lewis et al., 1974). The absorbed energy causes isomerization of the chromophore to 13-*cis*-retinal (Braiman & Mathies, 1980, 1982). Subsequent relaxation of this ground-state photoproduct is coupled to proton transport from the cytoplasm to the extracellular space. The produced proton gradient drives several energy-demanding metabolic reactions including ATP synthesis. Early investigations of the light-induced processes revealed the existence of intermediates termed J, K, L, M, N, and O, which are distinguishable by their visible absorption and which appear in this order (Lozier et al., 1975; Polland et al., 1986). A key intermediate is the M state in which the Schiff base becomes deprotonated (Aton et al., 1977; Marcus & Lewis, 1977) and protons are released from the protein. Decay of M is accompanied by reprotonation of the Schiff base and uptake of protons. The last step of the observed transitions is the re-formation of bacteriorhodopsin in its light-adapted state (BR_{568}). Since then, more elaborate models for the photocycle have been proposed. These include back-reactions between intermediates (Ames & Mathies, 1990; Váró & Lanyi, 1990, 1991; Váró et al., 1990) and parallel pathways (Diller & Stockburger, 1988). Beside the diversity of these reaction schemes, the occurrence of several intermediate states shows that proton translocation is a stepwise process. This implies the existence of at least one chemical group in the interior of the protein which is able to accommodate transiently a proton, before it is transferred to another site or released from the protein. Therefore, attention has been drawn to ionizable side chains in the transmembrane segments of BR which might serve the proposed function by providing a pathway for the positive charge through a hydrophobic environment. Particularly, the role of internal carboxyl groups has been investigated by Fourier-transform infrared (FTIR) and time-resolved infrared difference spectroscopy (Siebert et al., 1982; Engelhard et al., 1985; Eisenstein et al., 1987; Roepe et al., 1987). This technique is well suited for such studies, since the protonation state of carboxyl groups is monitored by characteristic infrared absorption bands. By observing absorbance changes which accompany the light-induced transition of BR_{568} to the M intermediate, it was found that at least one carboxyl group becomes protonated, causing an increase of the absorbance around 1760 cm^{-1} (C=O stretching vibration). A second protonation has been inferred from kinetic measurements of a spectral component at 1755

† This work was supported by the Deutsche Forschungsgemeinschaft (Az Si 278/6-1) and by the Swiss National Science Foundation.

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[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; FTIR, Fourier-transform infrared.

0006-2960/93/0432-5862$04.00/0

cm^{-1} of the absorbance increase (Engelhard et al., 1985). Incorporation of [4-^{13}C]Asp in the apoprotein shifts the maximum of the absorption band of the C=O stretching vibration(s) of M to about 1720 cm^{-1}, which provides evidence for one (two) Asp residue(s) to become protonated in M. Since there are four internal Asp residues (at positions 85, 96, 115, and 212) in BR, more selective methods for detailed assignments are desired. Recent progress has been achieved by FTIR studies on BR mutants. From these results, transient protonation changes of Asp-212 [protonation in M (Braiman et al., 1988a; Rothschild et al., 1990)], Asp-85 [protonation in M (Braiman et al., 1988a; Fahmy et al., 1992)], and Asp-96 [deprotonation in N (Gerwert et al., 1989; Bousché et al., 1991; Pfefferlé et al., 1991; Maeda et al., 1992)] have been inferred. However, NMR investigation of Asp resonances in BR_{568} and M shows that Asp-212 is deprotonated in both BR states (Metz et al., 1992a,b). On the other hand, it is difficult to prove a possible partial deprotonation by the NMR method since absolute intensities of the resonances are not accurately enough reproduced. Moreover, a fast equilibration between the protonated and deprotonated states might render the observation of protonated Asp-212 in M impossible. In contrast, the FTIR technique is in principle able to detect the actual protonation state in these cases.

Although site-directed mutagenesis seems to be a more specific approach to the assignment of infrared absorption bands as compared to isotopic labeling, severe implications can hamper the interpretation of spectroscopic data due to structural perturbations in the mutant protein. This is especially the case with amino acid substitutions for Asp-212. Effects of the substitution of different amino acids for Asp-212 on proton pumping and photocycle kinetics are described (Mogi et al., 1988; Stern et al., 1989; Otto et al., 1990; Needleman et al., 1991). For example, Asp-212Ala is not stable to light, and the Asp-212Asn mutant exhibits reduced pumping efficiency. Light-adaptation is sometimes abnormal when Asp-212 is substituted (Asp-212Asn and Asp-212Glu), and, unlike light-adapted BR of the wild-type, a considerable amount of 13-*cis*-retinal is always present in these mutants, including Asp-212Ala (Rothschild et al., 1990). Photoconversion of this isomer interferes with the absorbance changes of the all-trans state. Therefore, interpretation of the spectra cannot unambiguously rely on a direct comparison with the spectra from wild-type BR. As a consequence, no definitive spectral assignments in the range of symmetric and antisymmetric CO_2^- stretches of ionized carboxyl groups could be achieved (Rothschild et al., 1990). However, a tentative assignment of a vibration at 1738 cm^{-1} to the protonation of Asp-212 in M (Braiman et al., 1988a) was recently supported by an FTIR study of an Asp-212Ala mutant (Rothschild et al., 1990).

In order to circumvent such difficulties and to assign the protonation state(s) of Asp-212 in M, we apply a combination of fragment reconstitution and isotopic labeling in order to achieve more specific assignments. Protease V8 cleaves bacterioopsin in a cytoplasmic loop between Glu-166 and Val-167, yielding two fragments which comprise helices A–E (V-1) and helices F and G (V-2). These fragments can be reconstituted and regenerated with *all-trans*-retinal to the pump-active pigment (Sigrist et al., 1988). From the four internal Asp residues, Asp-212 is the only one present on V-2. This allows specific labeling of Asp-212 by reconstituting BR from V-1 fragments of unmodified BR and V-2 fragments of [4-^{13}C]Asp-labeled PM. With this experimental system which is devoid of structural and functional perturbations, it becomes possible to verify the conclusions gained from analysis of Asp-212 mutants. In addition, for the elucidation of the role of Asp-212 in the N intermediate, we have extended our study to conditions which favor its accumulation.

MATERIALS AND METHODS

Reconstitution of the Purple Membrane. PM was reconstituted from fragments V-1 and V-2 obtained by proteolytic treatment of BR with protease V8 essentially as described (Sigrist et al., 1988). After proteolysis of approximately 20 mg of BR, the fragments were lyophilized and delipidated by repeated extractions with chloroform/methanol (1:1 by volume) containing 0.1 M ammonium acetate [(1) extraction 2.5 mL; (2 and 3) extractions 1.25 mL each]. Combined extracts were chromatographed on Sephadex LH 60 (column dimensions: 70 × 4 cm) using the same solvent. Fractions containing V-1 were rechromatographed to achieve separation of uncleaved BR (Wüthrich & Sigrist, 1990). Pooled fractions of each fragment were concentrated by rotoevaporation (final volume approximately 6 mL). Fragments were transferred to SDS (sodium dodecyl sulfate) solutions by dialysis in 0.2% SDS/10 mM sodium phosphate buffer, pH 6.0 (3 times 1 L), followed by 0.2% SDS/10 mM sodium phosphate buffer, pH 11.8 (1 L) and 0.2% SDS/10 mM sodium phosphate buffer, pH 6.0 (1 L). Dialyzed samples were concentrated (Minicon B-15) to 3.5 mg of V-1/mL and 1.5 mg of V-2/mL, respectively (Sigrist et al., 1988).

The preparation of [4-^{13}C]Asp-labeled native BR has been carried out as described (Engelhard et al., 1985). An incorporation of about 40% can be inferred from the reduction of infrared absorbances which are caused by ^{12}C=O stretching vibrations of protonated Asp residues. Labeled fragments were combined with unlabeled complementary fragments (120 nmol of each) and adjusted to a final fragment concentration of 18 μM by addition of 0.2% SDS in 10 mM sodium phosphate buffer, pH 6.0 (final volume 6.6 mL). Fragments were mixed with DMPC/CHAPS [16 μL, DMPC (dimyristoylphosphatidylcholine), 8% w/v, and CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate], 3% w/v, in 10 mM sodium phosphate buffer, pH 6.0] and *all-trans*-retinal (156 nmol in 183 μL of ethanol) and incubated for 18 h at 23 °C. The lipid/protein/detergent mixture was completed by addition of purple membrane lipids (2 mg) and taurocholate (2.72 mg in 10 mM sodium phosphate buffer, pH 7).

Excess SDS was removed as described by dropwise addition of 320 μL of 4 M KCl (Popot et al., 1986). Precipitated potassium dodecyl sulfate was separated by centrifugation (1500*g*, 5 min at ambient temperature). Samples were then dialyzed against 5 changes (800 mL each) of 150 mM KCl/30 mM potassium phosphate buffer, pH 6.0. Upon removal of buffer salts by dialysis in H_2O (two changes, 1 L, 60 min), reconstituted purple membrane was collected by centrifugation (90 min, 100000*g*, 4 °C) and resuspended in 0.5 mL of H_2O. The procedure described yielded 4–5.5 mg of regenerated BR protein (25–30% of initially applied BR).

FTIR Measurements. About 200 μg of either reconstituted or native BR was dried under a gentle stream of nitrogen gas onto an AgCl window from a buffer-free suspension. The resulting PM film was hydrated by enclosing it with 2 μL of H_2O in an IR sample holder and cooled to 5 °C. Prior to FTIR measurements, the PM film was light-adapted by illumination with wavelengths above 455 nm within the spectrophotometer. To measure BR–M difference spectra, 256 interferograms were co-added, both under simultaneous illumination through an optical fiber cable with wavelengths

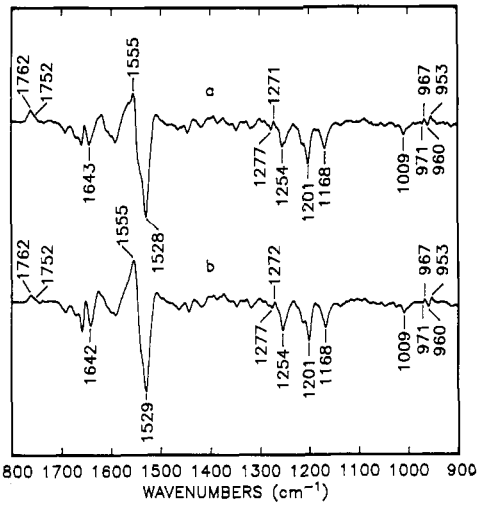

FIGURE 1: BR–M difference spectra of reconstituted BR (a) and native BR (b) at 278 K under continuous illumination with light of wavelengths above 455 nm. Absorption bands of the photoproduct show upward, those of the initial state (BR_{568}) downward. The magnitude of the absorbance change of the prominent ethylenic stretching vibration at 1528 cm^{-1} is about 0.008 absorbance unit.

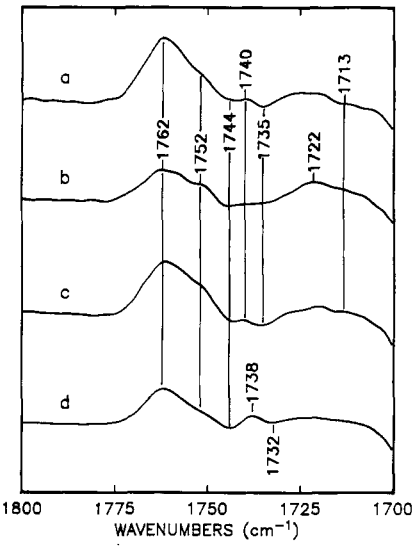

FIGURE 2: Absorbance changes during the BR–M transition in the spectral range of the C=O stretching frequency of protonated carboxyl groups. Experimental conditions and conventions as in the legend of Figure 1. (a) Reconstituted BR; (b) reconstituted [4-^{13}]C-Asp-V-1/V-2; (c) reconstituted V-1/[4-^{13}C]Asp-V-2; (d) native BR.

above 455 nm and in the dark. The averaged interferograms were Fourier-transformed, and from the single-beam spectra, the difference spectrum was formed. The signal to noise ratio of these difference spectra was improved by repetition of the measurements and averaging of the corresponding spectra. Spectral features of the N intermediate were obtained by the same procedure as described for the BR–M difference spectra, but 1 μmol of borate buffer (pH 9) had been added to the PM film and the temperature lowered to −5 °C. The described conditions were applied to both native and reconstituted BR.

Since only light-induced absorbance changes are measured in FTIR difference spectroscopy, the IR absorption of inactive V-1/V-2 fragments reduces the signal/noise ratio but does not produce its own bands in the spectra.

RESULTS

Measurements of the BR–M Transition. The absorbance changes which accompany the BR–M transition in reconstituted PM are in very good agreement with those typical of native BR. Figure 1 compares the BR–M spectra of both preparations in a spectral range which comprises absorption bands of the retinal chromophore as well as of the apoprotein. As is the convention for all difference spectra, negative bands belong to light-adapted BR (BR_{568}), and positive bands are caused by the respective photoproduct. Without going into details of band assignments for the chromophore and the protein, a brief summary of the typical features of the difference spectra is given to assess the degree of similarity between the native and reconstituted PM. The predominant negative band at 1527 cm^{-1} is caused by the C=C stretch vibration of the all-trans chromophore in BR_{568}. Its frequency is sensitive to the extent of π-electron delocalization along the conjugated double bonds of the chromophore which again depends on its electrostatic environment (Blatz et al., 1972). Therefore, the agreement in the C=C stretch frequency of reconstituted (Figure 1a) and native BR_{568} (Figure 1b) argues for the formation of a retinal binding site with a charge environment which matches essentially that of native BR.

Between 1300 and 1100 cm^{-1}, C–C single bond stretches coupled to C–H in plane bending vibrations contribute to most of the absorption bands. The coupling renders these bands sensitive to the chromophore geometry. The close correspondence of the pattern of the three (negative) absorption bands in Figure 1a with those in Figure 1b demonstrates that the chromophore in reconstituted BR_{568} is all-trans as in native BR_{568}. Small absorbance changes are visible between 1000 and 900 cm^{-1}, which are caused by hydrogen out-of-plane-vibrations of the chromophore. These modes are sensitive to single bond torsions (Fahmy et al., 1991). Since they are nearly identically reproduced in the reconstitution, the sterical constraints on the all-trans chromophore must be very similar to those of native BR.

Due to the deprotonation of the Schiff base in M and the concomitant decrease of charge alternation of the chromophore, only weak bands of the 13-cis chromophore in the photoproduct are observed. Only the C=C stretch of M at 1560 cm^{-1} is seen where it overlaps with absorbance changes from the amide II band. The latter causes a difference band which is more distinct in the reconstitution as compared to native BR. This might indicate an increase of structural flexibility upon cleavage of the covalent bond between Glu-166 and Val-167. However, the conclusions drawn from the well-reproduced chromophore bands in the reconstitution, which argue for the formation of almost the native chromophore binding site, are also corroborated by other difference bands of the protein. The band of BR_{568} at 1277 cm^{-1} is caused by Tyr-185 (Rothschild et al., 1986; Dollinger et al., 1986; Braiman et al., 1988b), which is in close vicinity to the chromophore (Henderson et al., 1990). The agreement between Figure 1a and Figure 1b in this band provides further support for the existence of nearly identical chromophore protein interactions during formation of M. Correspondingly, as it was shown for the native system, for the reconstituted preparation the protonation of at least one internal carboxyl group can be deduced from the absorbance increase at 1762 cm^{-1}. This is more clearly shown in Figure 2a in which the spectral region of the C=O stretching vibrations of protonated carboxyl groups is depicted. The shoulder at 1752 cm^{-1} and the neighboring pattern of small difference bands at 1744 (negative), 1738 (positive), and 1732 cm^{-1} (negative) of native BR shown in Figure 2d are also reproduced in the reconstituted fragments. The latter three bands are slightly up-shifted as compared with native BR and exhibit lower intensities. In contrast to native BR, the intensity of the shoulder at 1752

cm^{-1} exhibits small variations in different measurements of the reconstituted system.

The great similarity between the BR–M spectra of native BR and reconstituted V-1/V-2 fragments demonstrates that the gain of selectivity in isotopic labeling in the reconstituted system is not counterbalanced by a significant loss of structural and functional integrity as particularly encountered in Asp-212 mutants.

The influence of [4-^{13}C]Asp labeling of the proteolytic fragments on the BR–M spectra is also shown in Figure 2. In the spectrum of Figure 2b, the V-1 fragment is labeled. Compared with Figure 2a, the intensity of the band at 1762 cm^{-1} is lowered in favor of an increase at 1722 cm^{-1}. This agrees well with the expected isotopic shift of 40 cm^{-1} for the C=O stretching frequency of a protonated carboxyl group. Since the incorporation of label is considerably less than 100%, only part of the band is shifted to 1722 cm^{-1} whereas the remaining ^{12}C=O groups cause the decreased band at 1762 cm^{-1}. From the intensity ratio of both bands, an incorporation of the label of about 40% can be estimated. The shoulder at 1752 cm^{-1} and the adjacent negative band at 1744 cm^{-1} in Figure 2b appear more distinct as compared to Figure 2a. This is due to slightly different amounts of N in the M–N equilibria of the three isotopically distinguished preparations. Therefore, we will base the assignment of the 1752/1744-cm^{-1} difference band on BR–N spectra in which this band is a predominant feature (see below). However, the flat shape of the spectrum of Figure 2b at 1713 cm^{-1} as compared to the structured one in the other traces of Figure 2 suggests that part of the intensity at 1752 cm^{-1} has shifted to this region. The 1735/1740-cm^{-1} band pattern is also affected by the label in V-1. Both the positive lobe and the negative lobe of this structure are significantly reduced in Figure 2b as compared to Figure 2a. Therefore, all absorbance changes between 1720 and 1780 cm^{-1} are caused by aspartic acids located on the V-1 fragment, i.e., by Asp-85, -96, and -115. Correspondingly, no influence of isotopic labeling of Asp-212 should be observed in this spectral range. This is indeed confirmed by Figure 2c which shows the BR–M spectrum of the reconstitution with labeled V-2 fragment, i.e., BR with selectively labeled Asp-212. The main band at 1762 cm^{-1} and its shoulder at 1752 cm^{-1} are not affected by the label. Note particularly that also the small 1735/1740-cm^{-1} absorption bands have the same intensity in Figure 2c and Figure 2a. Therefore, the assignment of these bands to C=O stretches of protonated Asp(s) on V-1 is confirmed by the lack of an isotope shift upon labeling of the V-2 fragment.

The described isotope effects on the BR–M spectra of complementary labeled reconstitutions reveal that Asp-212 does not contribute to spectral changes in the range of stretching vibrations of protonated carboxyl groups during the BR–M transition. This rules out that any change of the protonation state of this residue occurs, since this would necessarily cause a negative or positive band sensitive to labeling. Therefore, Asp-212 must either be deprotonated (in BR_{568} and M) or be protonated, but in the latter case unaffected by the molecular changes which accompany the formation of M. In order to discern between these alternatives, we have investigated the spectral range of the symmetric CO_2^- stretching vibration between 1330 and 1430 cm^{-1} (Figure 3). The spectrum of the unlabeled reconstitution is presented in Figure 3a and for comparison that of native BR in Figure 3d. Incorporation of [4-^{13}C]Asp causes band shifts between 1370 and 1400 cm^{-1} irrespective of the label being located in V-1 (Figure 3b) or V-2 (Figure 3c). In Figure 3b, a negative band

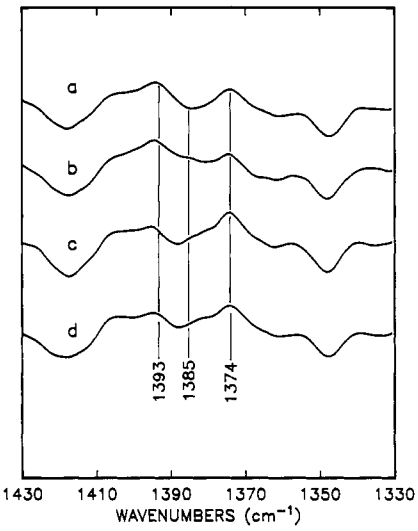

FIGURE 3: Absorbance changes during the BR–M transition in the spectral range of the symmetric CO_2^- stretching vibration of ionized carboxyl groups. Experimental conditions and conventions as in the legend of Figure 1. (a) Reconstituted BR; (b) reconstituted [4-^{13}C]Asp-V-1/V-2; (c) reconstituted V-1/[4-^{13}C]Asp-V-2; (d) native BR.

at 1385 cm^{-1} is shifted to lower frequency by 15 cm^{-1}, reducing the intensity near 1370 cm^{-1} as compared to Figure 3a. This shift renders the spectrum in Figure 3b more positive at 1385 cm^{-1} and reduces the lower frequency half of the positive band at 1374 cm^{-1}. Since the shifted band is negative, it must be assigned to BR_{568}. Therefore, a deprotonated Asp residue on V-1 is present in light-adapted BR and absorbs at 1385 cm^{-1}. Similarly, the influence of the label in V-2 (Asp-212) can be explained by the shift of a positive band (i.e., belonging to M) from 1393 to 1374 cm^{-1} (comparison of Figure 3c with Figure 3a).

The described assignments of C=O and CO_2^- stretches to Asp residues on either V-1 or V-2 allows us to determine the protonation state of Asp-212 in BR_{568} and in the M intermediate: Protonation changes of Asp-212 during the BR–M transition can be excluded, since we have shown that this residue does not contribute to any vibration between 1700 and 1800 cm^{-1} in the BR–M difference spectra. Therefore, the observation of an ionized Asp-212 (CO_2^- stretch at 1393 cm^{-1}) in the M intermediate implies also a deprotonated Asp-212 in BR_{568}.

Measurements of the BR–N Transition. Two reasons prompted us to extend our studies to the investigation of the BR–N transition: (1) We have tentatively assigned the absorbance increase in M at 1752 cm^{-1} as well as the BR_{568} vibration at 1744 cm^{-1} in the BR–M difference spectra to Asp residue(s) on V-1. The absorbance changes of these modes become more pronounced in the BR–N transition which is therefore well suited to verify the previous assignments. (2) It has been suggested that Asp-212 is part of a proton pathway from the cytoplasmic surface of the protein to the Schiff base, thereby facilitating the reprotonation of the Schiff base during the M–N transition (Braiman et al., 1988a; Rothschild et al., 1990, 1992). Therefore, the protonation state of Asp-212 in both intermediates is crucial for this functional model.

Figure 4 compares the BR–N difference spectrum of the reconstitution (Figure 4a) with that of the wild-type (Figure 4b). It is obvious that the close correspondence of both spectra reveals the structural and functional integrity of the reconstitution. Similar to BR–M difference spectra, the exact reproduction of HOOP modes between 900 and 1000 cm^{-1}

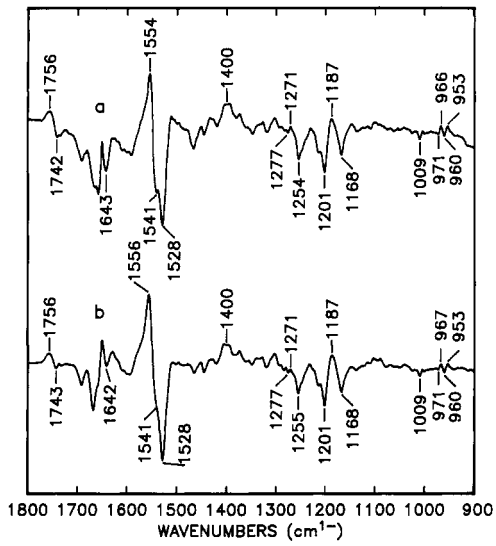

FIGURE 4: BR–N difference spectra of reconstituted BR (a) and native BR (b) at 278 K, 1 μmol of borate buffer (pH 9), under continuous illumination with light of wavelengths above 455 nm. Absorption bands of the photoproduct show upward and those of the initial state (BR_{568}) downward.

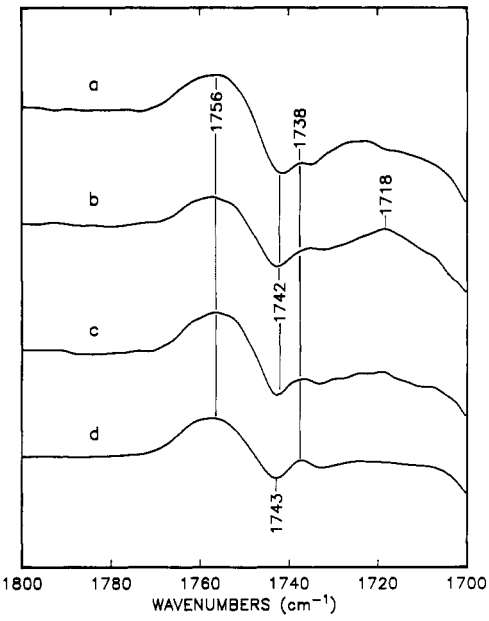

FIGURE 5: Absorbance changes during the BR–N transition in the spectral range of the C=O stretching frequency of protonated carboxyl groups. Experimental conditions and conventions as in the legend of Figure 4. (a) Reconstituted BR; (b) reconstituted [4-^{13}C]Asp-V-1/V-2; (c) reconstituted V-1/[4-^{13}C]Asp-V-2; (d) native BR.

provides further evidence of the re-formation of a nativelike chromophore binding site. After photoisomerization of the chromophore, single bond distortions additional to those inherent of the N intermediate would most likely cause alterations of the observed HOOP modes. The photoproduct band at 1187 cm^{-1} can serve as an indicator of N formation, since M does not cause any absorbance at this frequency. The relative intensity in both spectra indicates that approximately the same amount of N is formed under the same experimental conditions (pH 9). In Figure 4a, a small reduction of the water content during the time course of the measurements causes the drop of the base line between 1720 and 1600 cm^{-1}. Deviations from the BR–N difference spectrum of native BR are seen at 1554 cm^{-1}, where the shoulder of the negative band is more distinct in the reconstitution. In addition, a broad negative band between 1450 and 1500 cm^{-1} is seen in Figure 4a instead of a small absorbance change at this position for native BR in Figure 4b. A typical band of the N intermediate is observed around 1400 cm^{-1} where the intensity is considerably enhanced as compared to BR–M difference spectra (Figure 1). This band is well reproduced in the reconstituted preparation and of interest for the evaluation of the protonation state of the internal Asp residues since the symmetric stretching vibrations of ionized carboxyl groups absorb near this frequency.

In the following, we describe the effects of isotopic labeling of the proteolytic fragments on the BR–N difference spectra of the respective reconstitutions. In Figure 5, the spectral range of the C=O stretching vibrations of protonated carboxyl groups is depicted. The broad shape of the absorbance increase at 1756 cm^{-1} is caused by the superposition of the band at 1752 cm^{-1} caused by the N intermediate and the band at 1762 cm^{-1} typical of the M intermediate (Figure 2a,d), because both states contribute to the photostationary state. Figure 5b shows the difference spectrum of a reconstitution of which V-1 is labeled. It is obvious that the whole band pattern above 1740 cm^{-1} is reproduced as compared to Figure 5a. However, the intensity is reduced, demonstrating the involvement of Asp residues on V-1 (Asp-85, -96, -115) in these absorbance changes. The shifted band corresponding to the broad absorbance around 1756 cm^{-1} is seen around 1718 cm^{-1}. This confirms our assignment of the band at 1752 cm^{-1} (Figure 2) to the C=O stretching vibration of a protonated Asp on V-1 which is now much better observed since the amount of N is increased. Likewise, the negative band at 1742 cm^{-1} (which corresponds to that at 1744 cm^{-1} in BR–M spectra) has to be assigned to a vibration of an Asp residue on V-1 because it loses intensity in the labeled preparation. In agreement with the assignment of these absorbance changes to the V-1 fragment, no isotopic effect is observed in the spectral region between 1800 and 1740 cm^{-1} when V-2 (Asp-212) is labeled (Figure 5c). Correspondingly, no absorbance increase is observed around 1718 cm^{-1}. The specificity of the label with respect to Asp-212 allows the conclusion that Asp-212 does not alter its protonation state during the formation of N, which would cause a positive or negative band between 1700 and 1800 cm^{-1} sensitive to labeling of V-2. Since this has also been stated for the BR–M transition for which we could assign the symmetric CO_2^- stretching vibration of the ionized Asp-212 in M at 1393 cm^{-1}, we conclude that Asp-212 is ionized in N as well.

This conclusion can be verified by inspection of the spectral range of ionized carboxyl groups. The effects of isotopic labeling on the absorbance changes are shown in Figure 6. A comparison of Figure 6a with Figure 6b shows that the isotopic labeling of V-1 (b) reduces the intensity of the lower frequency half of the positive band at 1374 cm^{-1}. This parallels the result obtained with BR–M spectra (Figure 3a,b). Therefore, we assign this intensity change to the (negative) band of a symmetric $^{13}CO_2^-$ stretch at 1370 cm^{-1} which belongs to an Asp residue on V-1 in BR_{568}. However, the corresponding unshifted (negative) band which, in BR–M spectra, is located at 1385 cm^{-1} cannot be characterized by a comparable isotope effect at this position in the BR–N spectra. In addition, the positive band at 1394 cm^{-1} is slightly larger in Figure 6b than in Figure 6a. This argues for the involvement of additional CO_2^- groups in N as compared to M. A possible explanation will be given under Discussion.

In the case of the V-2-labeled preparation, we observe a close correspondence of the isotope shifts in BR–N spectra to

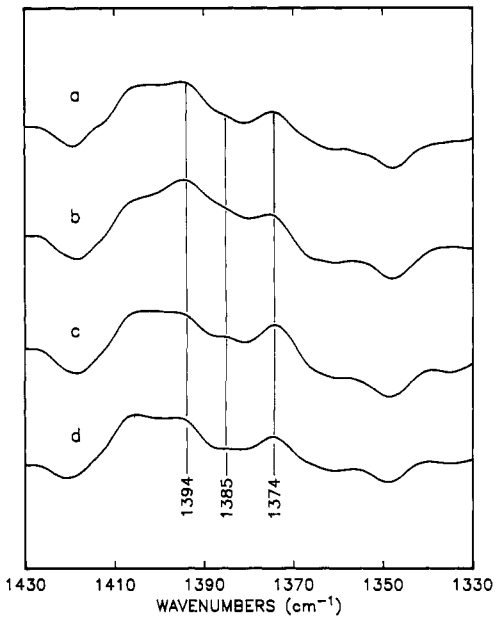

FIGURE 6: Absorbance changes during the BR–N transition in the spectral range of the symmetric CO_2^- stretching vibration of ionized carboxyl groups. Experimental conditions and conventions as in the legend of Figure 4. (a) Reconstituted BR; (b) reconstituted [4-^{13}C]Asp-V-1/V-2; (c) reconstituted V-1/[4-^{13}C]Asp-V-2; (d) native BR.

those described for BR–M spectra. A significant reduction of the intensity at 1394 cm^{-1} (Figure 6a) is caused by the labeling of Asp-212 (Figure 6c), and a concomitant increase at 1374 cm^{-1} can be attributed to the $^{13}CO_2^-$ stretching vibration of Asp-212. As in BR–M spectra, the label affects a positive band. Therefore, Asp-212 is also ionized in N.

DISCUSSION

We have investigated IR absorbance changes of aspartic acids of BR which occur during the formation of the photoproducts M and N. The identification of C=O stretches of protonated carboxyl groups and CO_2^- stretches of ionized residues has been achieved by [4-^{13}C]Asp isotopic labeling and detection of corresponding band shifts in FTIR difference spectra. These vibrations cause absorption bands between 1700 and 1800 cm^{-1} and around 1400 cm^{-1}, respectively. A reconstituted system has been used in which proteolytic fragments (V-1 and V-2 from protease V8 cleavage) were reassembled to form an active proton pump. The V-1 fragment (helices A–E) contains three internal Asp residues (85, 96, and 115). On V-2 (helices F and G), Asp-212 is the only internal aspartic acid. We have focused on the assignment of IR bands to Asp-212 which can be selectively labeled by reconstituting a V-2 fragment from a [4-^{13}C]Asp-labeled preparation with a V-1 unit of unmodified BR. From the reconstituted system, BR–M spectra are obtained which reproduce all features of those from native BR. Some deviations are observed for the amide II absorbance changes which are more pronounced in the reconstitution. This indicates a higher flexibility of the reconstituted protein which is caused by cleavage of the E–F loop. The structural changes during the BR–N transition seem to be more sensitive to this alteration than those of the M formation. But otherwise, the spectra of the reconstituted system are in excellent agreement with those of native BR, and the larger spectral differences are outside the range of the analyzed C=O and CO_2^- stretches.

A C=O stretching vibration at 1738 cm^{-1} in the M intermediate of wild-type BR has previously been assigned to a partial protonation of Asp-212 during M formation based on the lack of this band in Asp-212Asn and Asp-212Glu mutants (Braiman et al., 1988a). This has later been supported by an FTIR study on the Asp-212Ala mutant (Rothschild et al., 1990). In the reconstituted system, this band is observed at 1740 cm^{-1}. In contrast to these results, we could not detect any influence of 4-^{13}C labeling of Asp-212 on this band in BR–M difference spectra (Figure 2c). Therefore, we assign it to the C=O stretch of a protonated Asp residue on V-1. This is confirmed by a significant reduction of this band if V-1 is labeled (Figure 2b). Since also none of the other C=O stretches of protonated carboxyl groups between 1700 and 1800 cm^{-1} is affected by specific Asp-212 labeling, our results exclude any change of the protonation state of this amino acid upon M formation.

Investigation of the spectral range of symmetric CO_2^- stretches of the reconstitution between 1330 and 1430 cm^{-1} has helped us to determine the protonation state of Asp-212. An isotope-sensitive band is observed in M at 1393 cm^{-1}. It must be assigned to Asp-212 because it shifts to 1374 cm^{-1} when Asp-212 is labeled (Figure 3b). Therefore, Asp-212 is ionized in M. Due to the lack of a protonation change of this group upon M formation, we have to assume an ionized Asp-212 in BR_{568}, too. This agrees well with results from NMR studies (Metz et al., 1992a,b). However, the isotope incorporation causes a shift in the photoproduct band only, whereas no CO_2^- stretch can be assigned to Asp-212 in BR_{568} in the spectral range between 1330 and 1430 cm^{-1}. This argues for either an increase of the intensity of the carboxylate stretching mode during formation of M (instead of a frequency shift) or a frequency of this mode in BR_{568} outside the 1330–1430-cm^{-1} range. Since there is little information about the dependency of the infrared activity and isotopic shifts of the symmetric CO_2^- stretch, no interpretation in terms of the molecular environment of Asp-212 can be given. However, it is reasonable to assume an electrostatic interaction between the negatively charged carboxyl group and neighboring positive partial charges in BR_{568} and M for mutual stabilization in the interior of the protein. The three-dimensional model of BR (Henderson et al., 1990) suggests Tyr-185 to form a hydrogen bond with Asp-212. This is supported by FTIR measurements (Fahmy et al., 1991; Rothschild et al., 1990) and has been considered to be of functional importance for the reprotonation of the Schiff base (Rothschild et al., 1990). The absorbance changes of Asp-212 and Tyr-185 at 1393 and 1277 cm^{-1}, respectively, indicate that the isomerization of the chromophore is sensed by the hydrogen-bonded pair of amino acid residues. Correspondingly, a change in the hydrogen bond may be the reason for both difference bands. Although our results agree with the reported interaction between Asp-212 and Tyr-185, we exclude that the equilibrium position of the hydrogen bond is shifted to such an extent to Asp-212 that it can be considered as at least partially protonated. The band of M at 1762 cm^{-1} can serve as a good estimate for the IR absorption of the C=O stretching vibration of a single completely protonated carboxyl group. If the band at 1738 cm^{-1} was caused by a protonated Asp-212, it would represent approximately a 10% protonation as judged by the relative band intensities. As shown, the label incorporation and the signal to noise ratio in the presented spectra are both high enough to assign the small absorbance increase at 1738 cm^{-1} to an Asp residue on V-1. In this view, our data exclude a partial protonation of Asp-212 even as low as 10%.

The pattern of isotope sensitivity upon labeling V-2 is the same for BR–M and BR–N difference spectra: all absorbance

changes above 1700 cm^{-1} can be assigned to Asp residues on V-1, whereas labeling of Asp-212 causes a photoproduct band at 1394 cm^{-1} to shift to 1374 cm^{-1} (Figure 6). Therefore, our conclusions for the protonation state of Asp-212 in BR_{568} and in M can be extended to the N intermediate. This demonstrates that Asp-212 remains ionized in N. The prevalence of the charged carboxyl group of Asp-212 in M and N excludes the proposed direct role of this amino acid as a proton acceptor for the Schiff base reprotonation. During the M–N transition, the Schiff base becomes reprotonated (Fodor et al., 1988). However, according to our results, no transitional protonation/deprotonation of Asp-212 occurs in either photoproduct. The role of Asp-212 can better be described as contributing to a negatively charged environment of the retinal chromophore. From measurements of Asp-212 mutants expressed in halobacteria, it has been shown recently (Needleman et al., 1991) that Asp-212 takes part in the complex counterion (De Groot et al., 1990) although it is not the major determinant in color regulation. Besides that, the interaction with Tyr-185 may be important for the structural integrity of the retinal binding pocket which is impaired in corresponding BR mutants. This notion of the role of Asp-212 agrees with the results of a kinetic study of the Asp-212Asn mutant photocycle (Needleman et al., 1991) which suggest that there is no significant role of Asp-212 for the reprotonation of the Schiff base as judged from the pump-active (purple) form of the mutant.

Since we have excluded any contribution of Asp-212 to absorbance bands above 1700 cm^{-1}, the question arises which Asp residue(s) cause(s) the 1738-cm^{-1} vibration (1740 cm^{-1} in the reconstitution) previously assigned to a protonated Asp-212 in M. From the assignments of a 1740/1733- and a 1742/1748-cm^{-1} difference band in BR–L spectra to Asp-115 and Asp-96, respectively (Braiman et al., 1988a; Gerwert et al., 1989), we assign the absorption at 1738 cm^{-1} in M to Asp-115 [in the Asp-115Asn mutant, the band is hardly seen (Braiman et al., 1988a)]. The negative band at 1742 cm^{-1} which becomes pronounced in BR–N difference spectra and is sensitive to [4-^{13}C]Asp labeling of V-1 has been identified as being caused by Asp-96 (Braiman et al., 1988a; Gerwert et al., 1989).

In addition to the specific assignments of IR absorption bands to Asp-212, the reconstituted system allows some conclusions about the protonation changes of Asp residues on the V-1 fragment. From BR–M spectra (Figure 3), we have identified for BR_{568} a CO_2^- stretching vibration at 1385 cm^{-1}. It is known from FTIR and NMR studies (Braiman et al., 1988a; Engelhard et al., 1990) that Asp-96 and Asp-115 are protonated in BR_{568}. Therefore, we assign the band at 1385 cm^{-1} to the CO_2^- stretching vibration of the ionized Asp-85. This agrees with the notion that Asp-85 is the proton acceptor of the Schiff base deprotonation in M, giving rise to the absorption at 1762 cm^{-1}. However, the identified frequency of the carboxylate does not agree with a previously reported tentative assignment of the CO_2^- stretching vibration of either Asp-85 or Asp-212 to a band at 1417 cm^{-1}which was based on a comparison of an Asp-96Asn mutant with wild-type BR (Gerwert et al., 1989). Hence, Asp-85 as well as Asp-212 exhibits CO_2^- stretches below 1400 cm^{-1}.

For BR–N spectra, the influence of labeling V-1 is more difficult to interpret than for BR–M spectra. First, one has to take into account that under the measuring conditions a mixture of M and N is produced. Therefore, one has to discuss the possibility that bands in the difference spectrum are actually caused by the BR–M transition. Comparison of the difference spectrum shown in Figure 4 with a "pure" BR–N difference spectrum (Pfefferlé et al., 1991) indicates that about 70% of N is present in the mixture. Therefore, if bands in the difference spectrum of the mixture were due to the BR–M transition alone, a large intensity reduction would be observed as compared to the BR–M spectra. When Figure 6a,b is compared with Figure 3a,b, the only effect that is reproduced is the intensity decrease at 1370 cm^{-1}. Since it is comparable in magnitude to that observed in Figure 3b, it is concluded that the same molecular group is observed in the BR–N transition at this position. Therefore, we explain in analogy to BR–M spectra the intensity decrease by the $^{13}CO_2^-$ stretch of Asp-85 in BR_{568}. This observation is in agreement with the conclusion made by Braiman et al. (1991), Bousché et al. (1991), and Maeda et al. (1992) that Asp-85 is still protonated in N. The absence of a corresponding isotope effect at 1385 cm^{-1} in BR–N spectra can be explained by the absorption of an additional CO_2^- stretch in N which compensates the negative absorption of Asp-85 at 1385 cm^{-1}. Different isotope shifts of both groups would then account for the observation of the shifted band at 1370 cm^{-1}. Whether the additional ionized group on V-1 in the N state is Asp-96 as suggested (Gerwert et al., 1989; Bousché et al., 1991; Braiman et al., 1991; Otto et al., 1989) cannot be decided by our experiments. An additional ambiguity arises from the fact that there are four external Asp residues on V-1 (as compared to one on V-2). In contrast to the C=O stretches of protonated carboxyl groups which reveal themselves as internal groups by their high frequency, no such assignment can be made for the CO_2^- stretches. Particularly for the BR–N transition in which the reconstituted system exhibits larger structural changes than native BR, we cannot exclude that these Asp residues contribute also to isotope shifts in the difference spectra between 1330 and 1430 cm^{-1} in the V-1-labeled preparation. This might be an alternative explanation for the more complex pattern of isotope sensitivity in BR–N spectra than in BR–M spectra (the latter do not show any influence of V-1 labeling at 1394 cm^{-1}). This uncertainty in interpretation with respect to the effects of V-1 labeling on CO_2^- stretches does not affect the conclusions concerning Asp-212: (1) the lack of a change of the protonation state in M and N is solely based on the C=O stretches of the protonated residues which are all caused by internal residues; (2) the protonation state itself is inferred from CO_2^- stretches in BR–M spectra of the V-2-labeled preparation in which interference with external residues is very unlikely. In addition, only one isotope-sensitive CO_2^- stretch is observed in the BR–M and BR–N spectra of V-2-labeled preparations (shift from 1393 to 1374 cm^{-1}). This virtually excludes any contribution from external Asp residues because it is reasonable to assign this band to Asp-212 which is very close to the Schiff base and, therefore, should be affected by the all-trans to 13-cis isomerization during the formation of both intermediates. Note also that the frequency of the CO_2^- stretch of Asp-212 in M is the same as in N as deduced from V-2 labeling. This again argues against involvement, as compared to M, of additional external Asp residues in N adding to absorbance changes at this particular frequency.

We have pointed out that in BR–N spectra a broad absorbance increase around 1400 cm^{-1} is observed which lacks in BR–M spectra. It is tempting to consider this as a hint of the deprotonation of a carboxyl group during N formation as discussed by Braiman et al. (1991). However, the intensity of this band is approximately twice as large as the intensity of the identified CO_2^- stretches when the label incorporation of 40% is taken into account. This argues against CO_2^- as the major contribution to the 1400-cm^{-1} absorption. In fact,

this band is sensitive to H_2O/D_2O exchange (data not shown) and, therefore, most likely contains the N–H bending mode of the protonated Schiff base in N as a predominant vibration.

ACKNOWLEDGMENT

We thank W. Kreutz for his continuing support and stimulating discussions.

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