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Reconstitution of Bacteriorhodopsin from the Apoprotein and Retinal Studied by Fourier-Transform Infrared Spectroscopy†

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ABSTRACT: The reconstitution of the retinal-containing protein bacteriorhodopsin (BR) from the apoprotein and retinal has been studied by Fourier-transform infrared (FTIR) difference spectroscopy. 9-*cis*-Retinal which occupies the binding site but does not reconstitute the chromophore was used as "caged retinal". Photoisomerization to the *all-trans* isomer triggers the reconstitution reaction. Absorption bands in the FTIR difference spectra of the educt and product of the reaction could be assigned by comparison with a 9-*cis*-retinal FTIR spectrum or an FT-Raman spectrum of BR and due to band shifts observed upon deuterium exchange. Specific difference bands were assigned to the protonated carboxyl groups of D96 and D115 by use of the mutants D115N and D96N. Both aspartic acids are protonated also in the apoprotein with pK_a values above 10 and undergo a frequency shift toward higher wavenumbers indicating a more hydrophobic environment in the reconstituted protein. No indication was found for protonation changes of carboxyl groups or other protonatable residues when carrying out the reaction at pH values between 4 and 10. The pH-dependent protonation changes reported earlier [Fischer & Oesterhelt (1980) *Biophys. J. 31,* 139−146] therefore may be caused by protons in a hydrogen-bonded network. Mutations of E204, but not of D38 or E9, cancel proton uptake during reconstitution at high pH as well as proton release at low pH. It is concluded, that E204, without changing its protonation state itself, is part of a protonatable hydrogen-bonded network which changes its pK_a during reconstitution thereby causing the observed protonation changes.

Bacteriorhodopsin (BR),[1] the light driven proton pump in the purple membrane of *Halobacterium salinarum,* has been studied by a wide range of biochemical and physical techniques, giving insight into its structure and function (Mathies et al., 1991; Oesterhelt et al., 1992; Lanyi, 1993). Many studies focus on the molecular events during the light-triggered photocycle of BR for understanding the proton transport mechanism. Fourier-transform infrared (FTIR) difference spectroscopy was applied to investigate changes of the protein backbone, of the chromophore retinal, and of specific amino acid residues during the photocycle (Rothschild, 1992). Other reactions of BR have also been studied by FTIR spectroscopy, and the results contribute to a more detailed understanding of BR structure and function. For the purple−blue transition at low pH, protonation changes of water-exposed carboxyl groups have been detected (Gerwert et al., 1987). The photoreaction of BR490 which contains covalently bound 9-*cis*-retinal as chromophore (Maeda et al., 1980; Fischer et al., 1981) is accompanied by extensive alterations of carboxyl group vibrations (Chang et al., 1987). The blue to acid−purple transition induced by halide binding also affects bands of protonated carboxyl groups, presumably mainly D85 (le Coutre et al., 1995a). pH-induced structural changes of BR have been studied using the attenuated total reflection technique (Száraz et al., 1994).

In this work, FTIR difference spectroscopy was applied to investigate the reconstitution reaction of BR from bacterioopsin (BO) and retinal. This reaction has been studied biochemically in earlier work with respect to its mechanism (Schreckenbach et al., 1977; Gärtner et al., 1983), to retinal−protein interactions (Schreckenbach et al., 1977, 1978), and to protonation changes (Fischer & Oesterhelt, 1980). Two intermediates, characterized by their different λ_{max} values, have been observed in the reaction of *all-trans*-retinal with BO to form BR570:

$$\text{retinal} + \text{BO} \xrightarrow{a} 400\ \text{nm} \xrightarrow{b} 430/460\ \text{nm} \xrightarrow{c} \text{BR570}$$

Reaction a has been interpreted as fixation and ring-chain planarization of the retinal molecule in the binding site (Schreckenbach et al., 1977); reaction b is not yet understood in molecular terms. The Schiff base (SB) as the covalent linkage between retinal and protein is formed in step c, because on reduction under denaturating conditions the formation of retinyl-protein parallels the time course of BR570 formation (Gärtner et al., 1983), and because the retinal-containing membrane of mutant BR-K216A lacking this covalent linkage also exhibits an absorption maximum of 430/460 nm (Schweiger et al., 1994). Reaction c is the rate-limiting step of the overall reaction. The time constants of the reconstitution reaction are 71 s for the *all-trans* and 16 s for the *13-cis* isomer (Gärtner et al., 1983); at lower pH, the reaction is slower (Schreckenbach et al., 1978). Step c is restricted to *all-trans*- and 13-*cis*-retinal; the 11-*cis* isomer forms only a 430/460 nm chromophore, and 9-*cis*-

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[1] Abbreviations: BR, bacteriorhodopsin; FTIR, Fourier-transform infrared; BO, bacterioopsin; AM, apomembrane; PM, purple membrane; SB, Schiff base; PSB, protonated Schiff base; EC, extracellular; CP, cytoplasmic.

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retinal is fixed in the binding site, but planarization is blocked (Schreckenbach et al., 1978).

Since 9-*cis*-retinal is not able to reconstitute BR570, it can be used as a "caged retinal". Reconstitution is induced by blue light causing isomerization to the *all-trans* isomer which then undergoes the reaction to BR570. The photolabile educt can be either apomembrane (AM) with added 9-*cis*-retinal, or BR390, an AM containing noncovalently bound 9-*cis*-retinal which can be prepared from PM (Fischer et al., 1981):

$$\text{9-}cis\text{-retinal} + \text{AM (or BR 390)} \xrightarrow{h\nu} 400\text{ nm} \rightarrow 430/460\text{ nm} \rightarrow \text{BR570}$$

In solution, the overall reaction equation of the condensation leading to a protonated Schiff base is

$$RCH{=}O + H_2N{-}R' + H^+ \rightarrow RCH{=}N^+HR' + H_2O$$

The equation only allows for proton uptake at high pH (above pH 10, the amine, which is lysine 216 in the BR reconstitution reaction, is uncharged) or no proton participation at low pH (protonated amine).

The 9-*cis*-retinal trigger for reconstitution of BR was applied first by Fischer and Oesterhelt (1980) for determination of proton stoichiometries during reconstitution. While no protonation change was found at pH 6, a proton release was detected when carrying out the reaction at lower pH and a proton uptake at higher pH; the maximum effect was observed at pH 4 or pH 8, respectively:

$$\text{pH 4: BO} \rightarrow\rightarrow \text{BR} + H^+$$

$$\text{pH 6: BO} \rightarrow\rightarrow \text{BR}$$

$$\text{pH 8: BO} + H^+ \rightarrow\rightarrow \text{BR}$$

The fact of proton release at low pH and the course of proton stoichiometry as a function of pH was clearly in contrast to what solution chemistry would predict. Interestingly, a striking similar pH dependence of proton uptake and release was found for the relaxation of the photostationary state of BR (mainly M, but also N and O) back to the ground state (Garty et al., 1977; Fischer, 1979; Váró & Lanyi, 1990). In the case of BR reconstitution, the exact pH dependence of proton participation in the reaction of the protein was simulated by the assumption that the pK_a's of two protonatable groups shift from 4.6 to 2.8 and from 7.1 to 8.9 during reconstitution (Fischer & Oesterhelt, 1980). According to the model of the protonated SB being part of a proton donor−acceptor complex interacting with two protonatable groups (Fischer & Oesterhelt, 1979), it was suggested that these groups involved in the chromophore structure are identical with the residues changing the pK_a during reconstitution (Fischer & Oesterhelt, 1980), and could also be responsible for the net protonation changes of the protein in the M → BR reaction (Fischer, 1979). The low pK_a residue was discussed to be identical with the group responsible for the purple−blue transition at low pH, now shown to be D85 (Metz et al., 1992). A second candidate is D212 while the other intramembrane aspartic acids D96 and D115 which are protonated in BR570 at neutral pH (Braiman et al., 1988a; Gerwert et al., 1989) are candidates for the high-pK_a residues together with an unknown number of tyrosine, lysine, and arginine residues.

In the FTIR study reported here, the method of light-triggered reconstitution was applied to analyze protonation changes of the protein during BR reconstitution. For assignment of bands in the region of carbonyl stretching vibrations, the point mutated proteins D96N and D115N were used. The reaction at high and low pH was then investigated in order to eventually identify groups in the protein which change their pK_a. The detection of protonation changes during reconstitution by pH measurements was reproduced for BR wild type, and the same experiment was carried out for D38 and E204 mutants.

MATERIALS AND METHODS

Purple membrane sheets (PM) were isolated from *Halobacterium salinarum* S9 as described (Oesterhelt & Stoeckenius, 1974). Mutated PM's were isolated from transformed *H. salinarum* L33 cells containing the point-mutated BR D115N (kindly provided by J. K. Lanyi), D96N, E9Q, D38R, D38C, E204Q, or E204T, respectively. BR390 was prepared from wild-type or mutant BR as described (Fischer et al., 1981). AM was prepared according to Oesterhelt (1982). The AM suspension was washed several times with 2% BSA solution (Katre et al., 1981) until no further decrease of the retinal oxime absorption could be observed, and BSA was removed by subsequent washing with H_2O. 9-*cis*-Retinal was prepared by illumination of a solution of *all-trans*-retinal in acetonitrile with white light (50 W projector lamp, WG 335 filter) followed by HPLC separation of the isomers according to Denny et al. (1981). Measurements of pH changes during light-induced reconstitution were performed as described (Fischer & Oesterhelt, 1980), except for the amount of sample used, which was about 15−20 nmol in a 2 mL suspension.

For the FTIR measurements described in sections I and II under Results and Discussion, suspensions of either BR390 or AM to which an equimolar amount of 9-*cis*-retinal in 10 μL of 2-propanol had been added were adjusted to pH 6.5. Aliquots of the suspension (150 μL), containing 150−200 μg of protein, were dried, rehydrated, or equilibrated with D_2O vapor for about 12 h and placed in the FTIR spectrometer as described (Souvignier & Gerwert, 1992). The equilibration technique is sufficient for H/D exchange as these samples show identical effects as samples which have been washed several times with D_2O before (le Coutre and Gerwert, unpublished results). The sample holder was thermostated to 293 K. For the pH-dependent experiments (section III), the pH was adjusted in a 1 mL suspension of 10 μM BR390, 10 mM sodium phosphate or sodium borate buffer, and 0.1 M KCl. After centrifugation at 45000*g* for 3−4 h, the pellet was used without further drying. All these preparation steps were carried out under dim red light.

For photoisomerization, the sample was irradiated in the spectrometer with 300−500 laser flashes (repetition rate 20 Hz) of an excimer-pumped dye laser with 390 nm emission maximum, 10 mJ per flash. The FTIR spectra with 2 or 4 cm^{-1} resolution were obtained by recording 5−10 dark spectra in 128 single scans (taking about 6−12 min), followed by application of the laser flashes, at least 2 min reaction time (4−5 min for samples at pH 4), and then recording 5−10 spectra as before. The difference between

the first and the last dark spectrum served as a control of the base line quality. Comparison of the first spectrum after the delay time and the last spectrum showed no significant difference; therefore, no contributions of an intermediate state could be found in the first spectrum. The difference between the sums of the two series of spectra was calculated, giving the difference spectrum of the educt and product of the reconstitution. During the experiment, the sample was irradiated with yellow light (OG 530 filter) to prevent dark-adaptation of reconstituted BR570. This illumination does not enrich detectable amounts of M intermediate for wild-type BR at room temperature. In the case of D96N, additional blue light (GG 400 filter) was used to prevent M accumulation. Completeness of the reaction was controlled by recording UV/vis absorption spectra (Perkin Elmer Lambda 9 spectrometer) of the sample before and after reconstitution.

For recording the 9-*cis*-retinal FTIR spectrum, an isopropanolic solution of the isomer was evaporated on a CaF_2 window.

The FTIR difference spectra of educt and product of the reconstitution reaction are called "reconstitution difference spectra" in this paper. All these spectra are plotted with bands of BR390 (or AM with 9-*cis*-retinal) showing upward and bands of BR570, the reaction product, showing downward. This allows convenient comparison with FTIR difference spectra of photocycle intermediates. The reconstitution difference spectra compared in Figures 1−5 are normalized to the intensity of the ethylene band of spectrum 1B. Therefore, the ordinate scale in Figures 1−5 refers only to spectra 1B, 2B, 3A, 4A (pH 6), and 5B; the other spectra are scaled to comparable intensities.

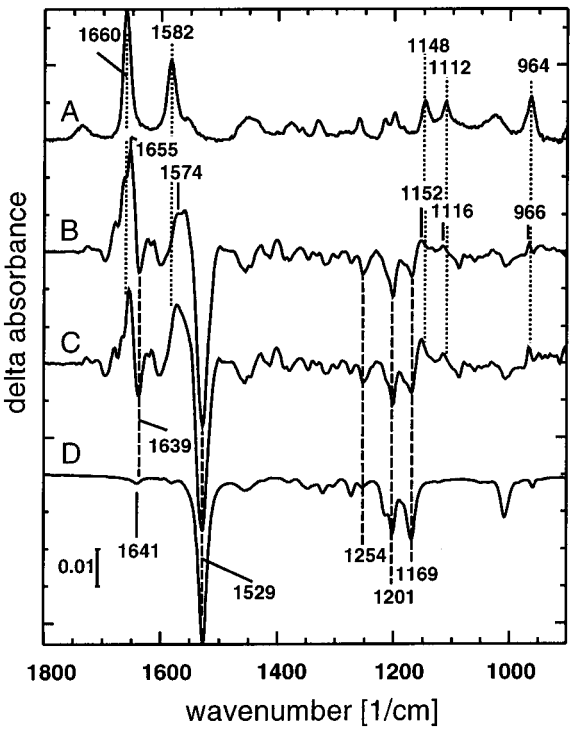

FIGURE 1: FTIR difference spectra of educt and product of the BR reconstitution reaction, and comparison with reference spectra. Dotted lines indicate bands of the reconstitution spectra which correspond to 9-*cis*-retinal vibrations, and dashed lines indicate chromophore bands of BR570 seen in the reconstitution spectra. (A) FTIR spectrum of 9-*cis*-retinal.[2] (B) FTIR difference spectrum of the reconstitution reaction BR390 → BR570. BR390 bands are showing upward, BR570 bands downward. (C) FTIR difference spectrum of the reaction AM + 9-*cis*-retinal → BR570. (D) FT Raman spectrum of PM (kindly provided by B. Hessling); spectral resolution 4 cm^{-1}.

RESULTS AND DISCUSSION

(I) BR390 − BR570 FTIR Difference Spectra. Illumination of a BR390 suspension with blue light yields BR570 which shows a 280/570 nm absorption ratio of 1.8. This indicates reconstitution of 95% of the BR molecules on the basis of an initial 280/570 nm ratio of 1.7 in the PM. If the reaction is carried out in a hydrated film used for FTIR experiments, 85−95% BR can be reconstituted from BR390, and 80% from AM with 9-*cis*-retinal added.

The FTIR difference spectra of the reconstitution reactions BR390 → BR570 and AM + 9-*cis*-retinal → BR570 in the range from 1800 to 900 cm^{-1} are shown in Figure 1B,C. The similarity of these two spectra proves that in both cases the same reaction is taking place. For band assignment, the reconstitution difference spectra are compared with an FTIR spectrum of 9-*cis*-retinal (Figure 1A) and an FT-Raman spectrum of the purple membrane (Figure 1D).

Some of the BR390 bands (positive peaks in Figure 1B,C, dotted lines) can be assigned to vibrations of 9-*cis*-retinal: the C═O stretching vibration at 1655 cm^{-1} in the reconstitution spectra (1660 cm^{-1} in the 9-*cis*-retinal spectrum), the ethylene band at 1574 (1582) cm^{-1}, C−C stretching vibrations at 1152 and 1116 (1148 and 1112) cm^{-1}, and the HOOP mode at 966 (964) cm^{-1}. At these band positions, particularly seen at 1574 and 1152 cm^{-1}, small differences are found between spectra 1B and 1C, indicating slightly different states of 9-*cis*-retinal in the two educt states. The lower reconstitution yield found for the 9-*cis*/AM preparation may be another aspect of this fact.

Compared to the 9-*cis*-retinal spectrum, the bands of single bond C−C stretches are shifted toward higher wavenumbers in the reconstitution difference spectra 1B and 1C, while the frequency of double bond vibrations (C═C and C═N) is lowered. This means that the protein stabilizes a retinal conformation with slightly weakened double and correspondingly strengthened single bonds when 9-*cis*-retinal is noncovalently fixed in the binding site.

Several bands of reconstituted BR570 (negative bands in Figure 1B,C, dashed lines) correspond to bands in the FT-Raman spectrum, which are assigned to chromophore vibrations in resonance Raman spectra of PM: most prominently in the double bond region are the ethylene band at 1529 cm^{-1} and the C═N stretching vibration of the protonated Schiff base (PSB) at 1639 cm^{-1} (1641 cm^{-1} in the FT-Raman spectrum); in the fingerprint region, for example, the band pattern of C−C stretches at 1254, 1201, and 1169 cm^{-1}. All these bands are characteristic for an *all-trans*-retinylidene chromophore with protonated SB (Smith et al., 1987).

The reconstitution difference spectrum (Figure 2B) shows similar features as the M−BR difference spectrum (Figure

[2] The band at 1740 cm^{-1} in the 9-*cis*-retinal spectrum may be caused by partial oxidation of the aldehyde group in this sample. Since the assignment and interpretation of the carboxyl bands in reconstitution difference spectra of BR are based on mutant proteins, an influence of this contamination can be excluded.

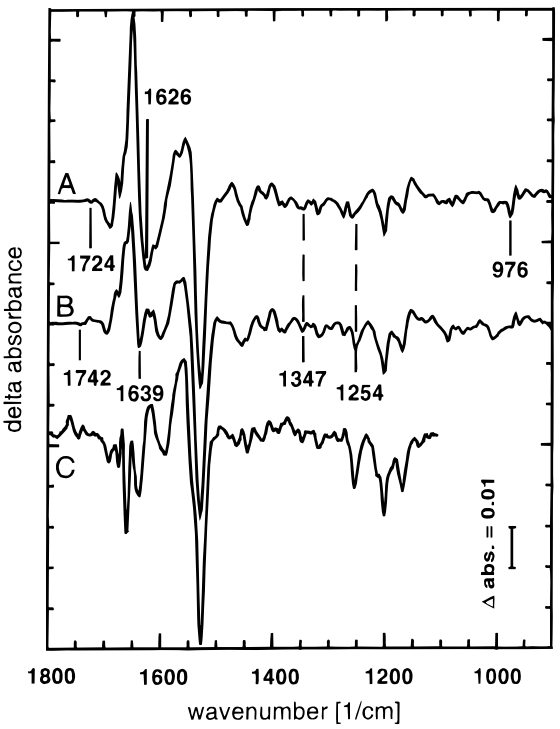

FIGURE 2: (A) FTIR difference spectrum of educt and product of the reconstitution of a D_2O-equilibrated sample; (B) H_2O-equilibrated sample, same spectrum as Figure 1B; (C) M−BR difference spectrum from Heβling et al. (1993); spectral resolution 4 cm^{-1}.

2C), in particular in the fingerprint region. This similarity in the region 1100−1500 cm^{-1} is attributed to the dominance of chromophore vibrations of the BR570 state in both cases. Band intensities of the chromophore with deprotonated SB (M state or 9-*cis*-retinal in the AM samples before reconstitution) are substantially lower than the intensities for the protonated form in the BR ground state.

Besides chromophore signals, the reconstitution difference spectra further reveal backbone changes of the protein and specific side group alterations. Changes of specific amino acid residues can be seen in the region above 1700 cm^{-1}; they will be discussed in detail in section II. The bands in the amide I and II regions indicating backbone changes overlap with the strong bands of the disappearing C═O group and the changing ethylene vibration. A comparison of the reconstitution difference spectrum (Figure 2B) with a M−BR FTIR difference spectrum (Figure 2C) shows that protein conformational changes, as far as they are reflected by amide band changes, are of the same order of magnitude during reconstitution and during the photocycle. Structural differences between PM and AM, on the other hand, have been investigated by CD spectroscopy (Gibson & Cassim, 1989) and IR linear dichroic spectral analysis (Draheim et al., 1991), revealing significant tilting of helices with respect to the membrane normal in the AM, while the secondary structure remains unchanged. Analysis of the amide I bands of BR and BO in deconvoluted IR spectra also proved that the distribution of secondary structural elements is essentially the same in both states (Cladera et al., 1996). This result is in agreement with the relatively smooth amide difference bands now observed in FTIR difference spectra of the reconstitution reaction. Since the amide I and II bands are mainly determined by the protein secondary structure (Krimm & Bandekar, 1986), the proposed tertiary structural changes may be hard to detect in the difference spectra. In this context, it is interesting, however, that the BR mutants K216A (Schweiger et al., 1994) and K216G (Friedman et al., 1994) show proton translocation activity when reconstituted with retinylidene ethylamine Schiff base, demonstrating that the structural features of BR which are important for its function still are conserved when the covalent linkage between retinal and protein is lacking.

The structural changes during reconstitution and M−BR relaxation give rise to amide difference bands of comparable intensities and also cause the same pH dependence of proton release and uptake but are not necessarily the same. One conspicuous difference is that the molecule shows only weak crystallinity in the state of apomembrane (Henderson, 1977; Fischer & Oesterhelt, 1980), but does not loose its capacity to form a two-dimensional crystal during the BR−M transition, since structural analysis of the molecule in the M state is possible by electron diffraction (Subramaniam et al., 1993). Crystallization could be mediated by only a small conformational change or alterations of only a few specific residues. Such minute motional changes may occur during reconstitution without causing extensive signals in the FTIR difference spectra. Therefore, detection and specific assignment of structural changes associated with the photocycle, with reconstitution, or with two-dimensional crystallization require complementation of FTIR experiments by other methods.

Some specific differences between the reconstitution difference spectra of D_2O- and H_2O-equilibrated samples (Figure 2A,B) deserve attention: (i) The band pattern around 1740 cm^{-1} is shifted to lower wavenumbers in the spectrum of the D_2O sample and weakened in its intensity, as known for the C═O stretching vibration of protonated/deuterated aspartic acids in BR (Engelhard et al., 1985). (ii) The 1639 cm^{-1} band shifts in D_2O to 1626 cm^{-1}, as in resonance Raman spectra (Smith et al., 1987), supporting the assignment to the C═N stretch of the PSB. (iii) The band pattern between 1345 and 1350 cm^{-1} is changed due to the shift of the NH in-plane rock vibration of the PSB to 976 cm^{-1} for ND, as known from resonance Raman data (Smith et al., 1987). (iv) The 1254 cm^{-1} band of the H_2O sample is sensitive to D_2O in the same manner as known from M−BR FTIR spectra (Engelhard et al., 1985).

(*II) 1700−1800 cm^{-1} Region: Protonated Carboxylic Groups*. In the 1700−1800 cm^{-1} region, C═O stretching vibrations of protonated carboxylic acids can be observed. The reconstitution difference spectrum shows a structure consisting of a 1728 cm^{-1} band in BR390 and bands at 1736 and 1743 cm^{-1} in BR570 (Figure 3A). Since it is known from FTIR investigations of the photocycle that the two aspartic acids D115 and D96 are protonated in the initial state BR570 (Braiman et al., 1988a; Gerwert et al., 1989), the reconstitution reaction was also carried out with the mutant proteins D115N and D96N. In the fingerprint and the amide region, no significant differences are found between the spectra of wild type and mutants (data not shown). Therefore, it can be concluded that these mutations do affect neither the chromophore nor the protein structure in BR390 or BR570. In particular, the reconstitution difference spectra of wild type and D115N agree in the region of amide I bands, while in M−BR difference spectra a specific effect of the mutation has been observed at 1640

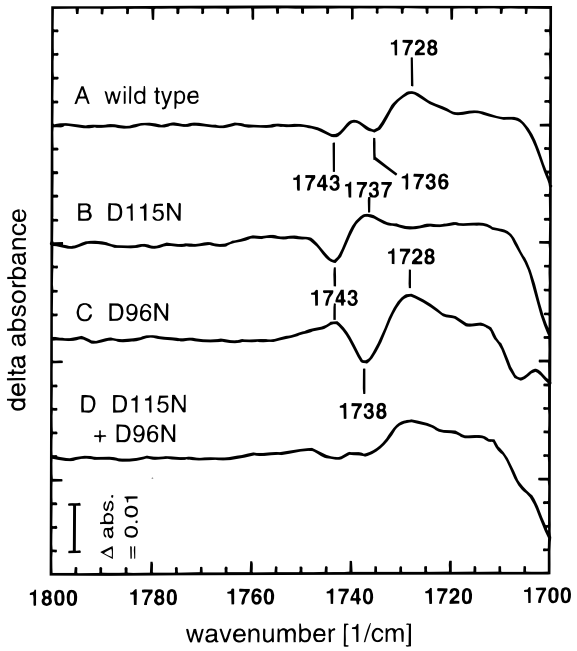

FIGURE 3: Region of protonated carboxylic groups in the reconstitution difference spectra, measured at pH 6.5. (A) Wild-type BR; (B) mutant D115N; (C) mutant D96N; (D) sum of spectra (B) and (C) exhibiting the wild type band structure; spectral resolution 2 cm^{-1}.

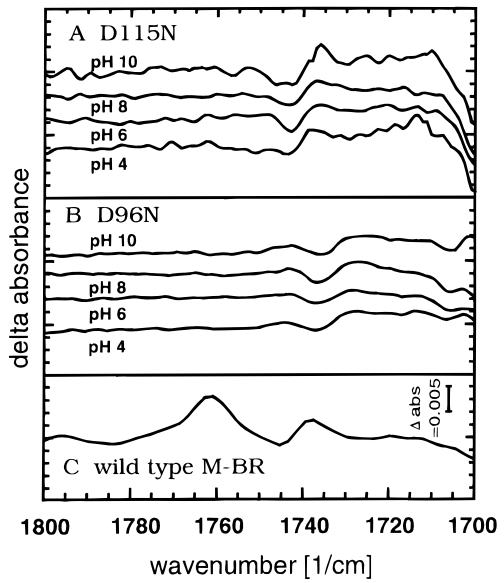

FIGURE 4: FTIR difference spectra of the reconstitution reaction at different pH values: region of protonated carboxylic groups. (A) Mutant D115N at pH 10, 8, 6, and 4, showing the D96 band; (B) mutant D96N at pH 10, 8, 6, and 4, showing the D115 band; spectral resolution 2 cm^{-1}; (C) M−BR difference spectrum (same as Figure 2C).

cm^{-1} (Sasaki et al., 1994). This conformational change in D115N compared with the wild type therefore occurs in the M intermediate, since the BR570 states are identical as demonstrated by the reconstitution spectra.

A strikingly different band pattern is found for the mutants in the region above 1700 cm^{-1}: The D115N spectrum, lacking the 1728 and 1736 cm^{-1} bands of the wild type, shows a difference band indicating a shift from 1737 cm^{-1} in BR390 to 1743 cm^{-1} in BR570 (Figure 3B). The D96N spectrum, on the other hand, lacks the 1743 cm^{-1} band of wild type; it consists of a 1728/1738 cm^{-1} difference band and a small, but reproducible band at 1743 cm^{-1} belonging to BR390 (Figure 3C). Comparison of the three spectra Figure 3 A−C shows that the wild-type band structure is a superposition of two difference bands caused by D96 and D115, which can be seen individually in the spectra of D115N or D96N, respectively. Since difference bands are seen for the two aspartic acids, consisting of absorption signals from the educt and the product of the reconstitution reaction, it can be concluded that D96 and D115 are protonated in the AM as well as in the PM; this will be discussed in detail in section III. As a control that only these two aspartic acids contribute to the absorption changes, Figure 3D shows the sum of the D115N and D96N spectra which restores the band structure of the wild-type spectrum. Therefore, we interpret the 1743 cm^{-1} band in the D96N spectrum as part of a broad D115 absorption in BR390 which may result from a heterogeneous environment of D115 in the AM.

Both aspartic acid C═O bands shift toward higher wavenumbers during reconstitution. This can be interpreted as change toward a more hydrophobic environment and decreased interaction of the C═O bond of the carboxyl group with hydrogen bonding partners (Gerwert et al., 1989; Sasaki et al., 1994). Both residues are located in the hydrophobic region toward the cytoplasmatic (CP) side, above the retinal moiety in the model of the BR structure (Henderson et al., 1990; Grigorieff et al., 1996). The shift of the D96 and D115 bands shows that the structure in the AM is less hydrophobic and rigid in this region, allowing heterogeneity in the D115 environment, while it becomes more hydrophobic and better defined during reconstitution.

As shown in this section, the reconstitution reaction provides the possibility of assigning bands in the FTIR spectra of the initial state BR570, independently of photocycle experiments, which always have to deal with the problem of mixtures of intermediates produced by light activation of BR570 (Heβling et al., 1993).

(*III) FTIR Difference Spectra of the Reconstitution Reaction at Different pH Values*. According to a model proposed by Fischer and Oesterhelt (1980), two protonatable amino acid residues are changing their pK_a values during reconstitution and become protonated at higher or deprotonated at lower pH, respectively. Therefore, the reconstitution reaction was carried out at different pH values. Differences between high- and low-pH spectra, correlating with the proton uptake or release (see pH traces for wild-type BR in Figure 6), then could be used as indicators for the specific groups changing their protonation state.

At first, the spectral range above 1700 cm^{-1} is of special interest because protonation changes of aspartic acids can be observed in this region. The mutants D96N and D115N are used for experiments at pH 4−10, since their spectra are less complex in the carbonyl region than that of wild type (see section II). In the resulting spectra (Figure 4A,B), difference bands assigned in the previous section to D115 and D96 are seen over the complete pH range. Besides these, no additional bands occur, neither at high nor at low pH, indicating that no carboxyl group is changing its protonation state during reconstitution at pH values from 4 to 10. In particular, the two aspartic acids D96 and D115 are protonated in the AM as well as in the PM with a pK_a value higher than 10. It is known from pH-dependent FTIR measure-

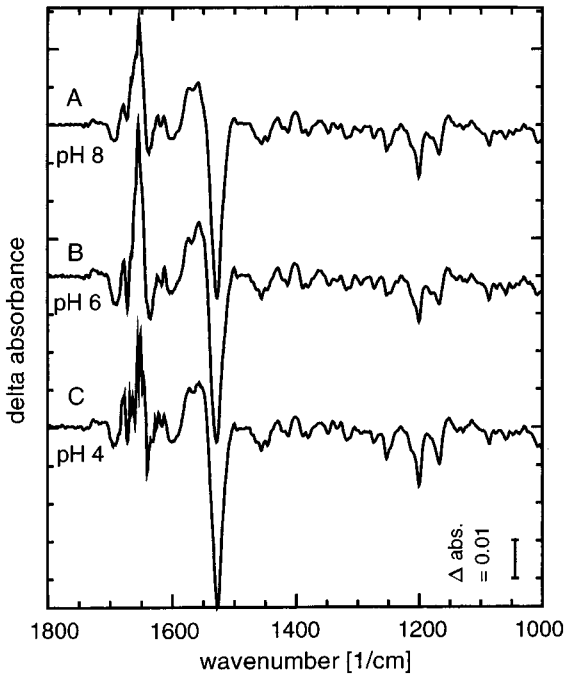

FIGURE 5: FTIR difference spectra of the reconstitution reaction at different pH values: wild-type BR at pH 8 (A), pH 6 (B), and pH 4 (C); spectral resolution 2 cm^{-1}.

ments (Száraz et al., 1994) that in the PM D115 does not deprotonate up to pH 12 and D96 exhibits an apparent pK_a of 11.5 which reflects a protein conformational change making D96 accessible to the external medium. The results of the reconstitution experiments show that also in the AM the pK_a of these aspartic acids is unusually high, even though the environment of these residues is less hydrophobic than in the PM. Figure 4C shows the carbonyl region of a M−BR difference spectrum of wild-type BR as calculated by factor analysis and decomposition (Heβling et al., 1993). The intense band at 1762 cm^{-1} is caused by protonation of D85 (Braiman et al., 1988a); no such band is found in the reconstitution spectra. D85 and D212, being deprotonated in the PM, are also deprotonated in the AM with pK_a values lower than 4, as can be concluded from the lack of additional signals in the reconstitution difference spectra at lower pH. The band pattern around 1740 cm^{-1} in the M−BR spectrum corresponds to the position of the difference bands assigned to D96 and D115 in the reconstitution difference spectra. Indeed, this M−BR difference band is composed of D96 and D115 bands (Sasaki et al., 1994).

In order to check for bands indicating protonation changes of other groups besides carboxylic acids, the reconstitution difference spectra from 1800 to 1000 cm^{-1} for wild-type BR at pH 4, 6, and 8 are shown in Figure 5. Possible candidates for protonatable groups in BR would be tyrosine, arginine, and lysine residues. Tyrosine residues are discussed to undergo protonation changes during the photocycle (Roepe et al., 1987); in particular, a 1277 cm^{-1} band in the M−BR FTIR difference spectrum has been assigned to Y185 (Braiman et al., 1988b; Sonar et al., 1994). Arginine absorptions have not been assigned in BR FTIR difference spectra up to now; in the closely related light-driven chloride pump halorhodopsin, however, a specific signal of R108 (corresponding to R82 in BR) has been identified at 1695 cm^{-1} (Rüdiger et al., 1995), and further arginine signals are supposed to occur at about 1620 and 1180 cm^{-1} (Braiman et al., 1994). Concerning lysine residues, a specific influence of K216 was found at 1348 cm^{-1} (Gat et al., 1992); in H_2O solution, the side group of lysine exhibits bands at 1630 and 1526 cm^{-1} (Venyaminov & Kalnin, 1990). In all these regions no differences could be found between the reconstitution spectra at different pH values (Figure 5). Tyrosines, arginines, or lysines, nevertheless, cannot be excluded as clearly as the internal aspartates being responsible for the protonation changes observed by Fischer and Oesterhelt (1980). Identification of bands corresponding to protonation changes may be difficult, because in the fingerprint region the reconstitution difference spectra are dominated by the chromophore bands of BR570, and the signals of the proposed groups may be small.

(*IV) A Hydrogen-Bonded Network Changes Its Protonation State during Reconstitution*. An alternative explanation for the protonation changes not assuming specific residues changing their protonation states is the presence of an internal hydrogen bonded network consisting of polar side groups, protein backbone, and bound water molecules in the protein. Such networks can stabilize a quasi-delocalized proton which does not belong to a single and identifiable residue, as shown by FTIR spectroscopy of model systems (Zundel, 1994). The presence of bound water in BR, especially near the chromophore binding site, and indications for its specific role have been demonstrated by resonance Raman spectroscopy (Hildebrandt & Stockburger, 1984), by neutron diffraction (Papadopoulos et al., 1990), and by FTIR difference spectroscopy in the region of the OH stretch mode above 3500 cm^{-1} (Maeda et al., 1992; Fischer et al., 1994). Experimental evidence for proton transfer via a hydrogen bonded network between cytoplasm and the Schiff base was found by analysis of IR continuum absorption changes (le Coutre et al., 1995b). In this study, protonation changes were observed which are caused by the network but not by specific side chains of the protein. The attempt to identify reproducible continuum absorption changes in the reconstitution difference spectra failed because of the low intensity of these bands compared to the noise level. This problem could not be overcome by an averaging procedure as is possible in photocycle experiments (le Coutre et al., 1995b).

Recently glutamic acid 204 was proposed as the extracellular (EC) proton release group in the proton transport cycle on the basis of molecular dynamics calculations (Scharnagl et al., 1995) and mutational analysis (Brown et al., 1995). Instead of the carboxyl group of E204 itself, a hydrogen-bonded network stabilized by E204, bound water, and other residues could be responsible for proton release (le Coutre & Gerwert, 1996; Rammelsberg, Kuhn, and Gerwert, unpublished results). A cytoplasmic (CP) surface aspartic acid, D38, is involved in the reprotonation of the Schiff base in the M to N transition, but no FTIR signals indicating D38 protonation changes could be identified (Riesle et al., 1996). It might well be that D38 plays a similar role for proton reuptake by BR as E204 does for proton release, both residues being components of hydrogen-bonded networks which create the CP reprotonation or the EC deprotonation pathway, respectively.

To test for the role of these residues in the BR reconstitution reaction, E204 and D38 mutants were used for measurement of protonation changes according to Fischer and Oesterhelt (1980) by illuminating a BR390 suspension with blue light and recording pH changes. While no protonation

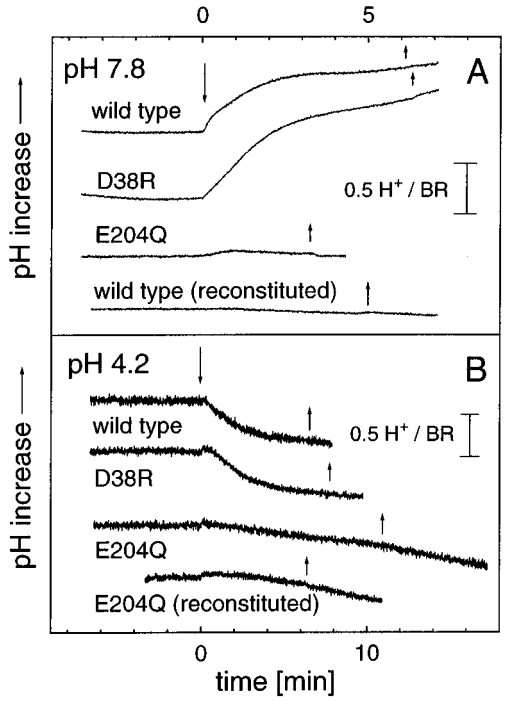

FIGURE 6: Traces of pH changes during the light-induced reconstitution reaction BR390 → BR570, starting at pH 7.8 or pH 4.2, for wild-type BR and the mutants D38R and E204Q. Constant drifts before illumination were eliminated by subtraction of a straight line from each data set. As a control, the signal of completely reconstituted samples (wild type at pH 7.8, E204Q at pH 4.2) shows only light-induced artifacts. Onset (time 0) and end of illumination are indicated by arrows. For calibration of the pH signal, the pH change due to addition of a known amount of HCl was measured, and the amount of reconstituted BR570 was determined from the absorption change at 570 nm before and after the reaction. The observed pH change was in the range of 0.05 pH unit at pH 7.8 and 0.01 unit at pH 4.2.

change was found at pH 6 for wild-type BR or the mutants (data not shown), at pH 7.8 light-induced alkalinization corresponding to proton uptake by the protein was observed in the case of wild type and the mutants D38R (Figure 6A) and E9Q (not shown). As a control, the completely reconstituted wild-type BR570 sample does not show a blue light-induced protonation change (Figure 6A, lowest trace), indicating that the pH changes are indeed caused by the reconstitution reaction from BR390 to BR570. At pH 4.2, a pH decrease corresponding to proton release is observed for wild type and D38R (Figure 6B) or D38C (not shown). Completeness of the reconstitution was checked by recording absorption spectra in the chromophore region. The stoichiometry of about 0.5−0.7 proton per BR, determined by calibrating the pH change with HCl and measuring the amount of reconstituted BR570 photometrically, is in accordance with the value found by Fischer and Oesterhelt (1980).

In contrast to wild type and D38R, no protonation change was found for E204Q (Figure 6A, third trace) or E204T (not shown) at pH 7.8. The lack of proton uptake during reconstitution at high pH is specific for mutation of residue 204 on the EC surface of BR, because another EC surface mutant, E9Q, behaves as wild-type BR. At pH 4.2, E204Q does not show a clear-cut proton release signal as wild type and D38R; only a slightly changed constant drift is observed upon illumination, which is probably a light-induced artifact, since the same light-induced drift is found for the completely reconstituted E204Q sample (Figure 6B, lowest trace) and in the wild-type and D38R traces after reconstitution (Figure 6B). In the sensitivity range of the measurements (changes of about 0.05 pH unit at pH 7.8 and 0.01 unit at pH 4.2), such drift changes could not be completely avoided. Therefore, the E204Q mutation also cancels the proton release at low pH, demonstrating that both effects, at pH 8 and 4, depend on E204. However, for two reasons we think it is not the glutamic acid 204 itself which changes its protonation state upon reconstitution: first, the same residue cannot take up a proton at pH 8, but release it at pH 4; and second, no additional signals indicating protonation changes of a carboxyl group were found in the reconstitution spectrum at pH 8 or 4 (see Figure 4). A reasonable explanation for the observed effect is that E204 is part of a protonatable network between the Schiff base location and the EC surface which has different pK_a's in the AM and the PM. This network might include water molecules, backbone peptide groups, and the side chains of D85, R82, Y57, and E204 (Scharnagl et al., 1995; Brown et al., 1995; Govindjee et al., 1996), and it would couple their pK_a's, not allowing identification of specific groups changing their protonation state. The corresponding network in the CP channel, in which D96 and D38 are participating (le Coutre et al., 1995b; Riesle et al., 1996), does not contribute to the protonation changes, since D96 gives rise to identical signals in the FTIR reconstitution spectra at pH 4 and 8 (Figures 4 and 5), and since the D38R mutation does not influence proton uptake or release (Figure 6). Therefore, we conclude that the EC network has the capacity of binding two protons, one uptake/release taking place at low pH where the network is protonated in the AM, but deprotonated in the PM, and one at high pH, the network being deprotonated in the AM and protonated in the PM. According to the description of the effects at high and low pH as pK_a shifts (Fischer & Oesterhelt, 1980), the two pK_a's get more separated during reconstitution, thereby increasing the pH range with one proton in the network. Since the difference between M and BR570 with respect to the protonation state resembles the difference between AM and PM (see the introduction), apparently the state characterized by more separated pK_a's depends on the existence of a positive charge at the place of the Schiff base nitrogen. The other state is correlated with either an empty, a 9-*cis*-retinal-containing, or a deprotonated Schiff base containing binding site. The correlation between reconstitution and photocycle is supported by the fact that E204 regulates the protonation state of the network and the protonation changes during reconstitution (Figure 6) as well as the proton release in the photocycle (Brown et al., 1995). The lack of proton uptake during reconstitution of E204Q at pH 8 may result in an initial protein state that contains one proton less in the EC network than the wild type does, and it may therefore be directly correlated with the lack of proton release in the L to M transition found for this mutant (Brown et al., 1995).

(*V) Conclusion*. With the help of the 9-*cis* isomer as a "caged" retinal, photorelease of *all-trans*-retinal allows the investigation of molecular changes during the reconstitution of the BR chromophore from retinal and the apoprotein by use of FTIR difference spectroscopy. In particular, specific signals of the aspartic acid side chains D96 and D115 could be assigned. No protonation change of carboxyl groups, but changes in the environment of D96 and D115, was found. Mutation of E204 to Q or T cancels the proton uptake during

reconstitution at pH 8 and the proton release at pH 4, but no protonation change of the E204 carboxylic group itself was observed in the FTIR spectra. We conclude that the unusual pH profile of the reconstitution reaction which resembles the pH-dependent protonation changes in the M to BR reaction is caused by pK_a changes of a hydrogen-bonded network connecting the PSB with the extracellular surface of the BR molecule, which are regulated by E204.

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