Time-Resolved Fourier Transform Infrared Study of Structural Changes in the Last Steps of the Photocycles of Glu-204 and Leu-93 Mutants of Bacteriorhodopsin†

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ABSTRACT: The last intermediate in the photocycle of the light-driven proton pump bacteriorhodopsin is the red-shifted O state. The structure and dynamics of the last step in the photocycle were characterized with time-resolved Fourier transform infrared spectroscopy of the mutants of Glu-204 and Leu-93, which accumulate this intermediate in much larger amounts than the wild type. The results show that E204Q and E204D give distorted *all-trans*-retinal chromophore like the O intermediate of the wild type. This is simply due to the perturbation of the proton acceptor function of Glu-204 in the O-to-BR transition in the Glu-204 mutants. The corresponding red-shifted intermediates of L93M, L93T, and L93S have a 13-*cis* chromophore like the N intermediate of the wild type, as reported from analysis of extracted retinal [Delaney, J. K., Schweiger, U., & Subramaniam, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11120−11124]. In spite of their different chromophore structures from the O intermediate, the red-shifted intermediates are similar to the O intermediate but not to the N intermediate of the wild type with respect to structural changes in the peptide carbonyls. The structural changes around Asp-96 in the N intermediate are completely restored also in the red-shifted intermediates of the Leu-93 mutants like in the O intermediate. These results imply that the protein structural changes in the last step proceed regardless of thermal isomerization of the chromophore. Time-resolved Fourier transform infrared spectroscopy with the Glu-204 mutants suggests that the response of Asp-204 (Glu-204 in the wild type) to the protonation of Asp-85 during formation of the M intermediate, which results in proton release, is slow and may occur through structural changes.

Bacteriorhodopsin functions as a light-driven proton pump in *Halobacterium salinarium*. The retinal chromophore is covalently bound to Lys-216 through a protonated Schiff base and can assume both *all-trans* and 13-*cis* configurations. Light adaptation changes the 13-*cis*,15-*syn* form to *all-trans*,15-*anti*. Additionally, photoisomerization of the *all-trans*-bacteriorhodopsin (BR)[1] to 13-*cis*,15-*anti* triggers a cyclic reaction that comprises a series of intermediates. Each intermediate was originally identified by visible absorption spectroscopy as reflected in their subscripts with the absorption maxima (K_{600}, L_{550}, M_{412}, N_{565}, and O_{640}).[2] This means that the chromophore−protein interaction, reflected in the visible spectrum, is distinct in each of these photointermediates.

Extensive spectroscopic studies have identified (i) the chromophore structure, (ii) the protonation state of the residues that contribute to proton pumping, and (iii) the protein structure of the intermediate states [for reviews see Mathies et al. (1991), Oesterhelt et al. (1992), Rothschild (1992), Lanyi (1993), Krebs and Khorana (1993), Ebrey (1993), and Maeda (1996)]. The K-to-L transition precedes the primary proton transfer from the Schiff base to Asp-85 with accompanying structural changes in the protein and internal bound water. The proton transfer in the L-to-M process causes proton release from Glu-204 to the extracellular aqueous phase (Brown et al., 1995). The protonated Asp-85 and the unprotonated Glu-204 thus formed appear to persist until the final step in the photocycle. The M-to-N process is accompanied by proton transfer from Asp-96 to the Schiff base. The greatest structural changes of the protein in the photocycle are observed in the M_N and N intermediates (Sasaki et al., 1992; Kamikubo et al., 1996). Proton uptake from cytoplasmic aqueous phase to Asp-96 and thermal reisomerization from 13-*cis* to *all-trans* occurs in the step subsequent to the formation of the N intermediate, and the final proton transfer from Asp-85 to Glu-204 resets the original structure of BR.

Unlike the case of visual rhodopsin, the initial state recovers thermally in the photocycle of BR, which allows it to turn over rapidly as required for an energy converter rather than a signal transduction device. Therefore, it is important to elucidate the molecular mechanism for how bacteriorhodopsin resets the system. However, in comparison with the earlier events, the structural changes in the late processes

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[1] Abbreviations: BR, bacteriorhodopsin (or its mutants) with *all-trans* chromophore; HOOP, hydrogen out-of-plane; TR-FTIR, time-resolved Fourier transform infrared.

[2] These subscripts originate from Váró and Lanyi (1991).

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(N → O → BR) are poorly understood. One of the reasons is that the O intermediate is not accumulated in large amounts in the photocycle of the wild type, and kinetically it is not well separated from the N intermediate. The following three differences between the N and O intermediates had been noted: (i) the visible spectrum of the O intermediate is red-shifted (difference absorption maximum ~640 nm) rather than blue-shifted as for the N intermediate, (ii) the chromophore configuration is 13-*cis* in N and *all-trans* in the O intermediate (Smith et al., 1983), and (iii) under usual conditions Asp-96 is deprotonated in N and protonated in the O intermediate (Hessling et al., 1993). According to these properties, the N-to-O process is usually thought to be accompanied by proton uptake from the cytoplasmic aqueous phase and thermal reisomerization of the retinal chromophore from 13-*cis* to *all-trans*. However, objections have been raised against this simple model. For instance, an N intermediate which has already taken up a proton [called $N^{(0)}$] has been described in the wild type at alkaline pH (Zimányi et al., 1993) and in some mutants (Brown et al., 1994).

In studying the late steps of the photocycle, mutants that produce long-lived intermediates can be useful. A number of such mutants have been reported. One is Y185F at alkaline pH (Bousché et al., 1992). In many respects its FTIR spectrum is in accordance with that of the O intermediate in the resonance Raman spectrum of the wild type (Smith et al., 1983) except for exhibiting a single hydrogen out-of-plane (HOOP) vibrational band, at 956 cm^{-1}, rather than several. The O intermediate of Y185F therefore has an *all-trans* chromophore which is distorted differently from that of the wild type. Mutants of Leu-93 (L93A and L93T) are other examples. The last intermediate of their photocycles is long-lived but less red-shifted (difference absorption maximum around 600 nm, with the initial state blue-shifted from the wild type) (Subramaniam et al., 1991). Recently, the chromophore of this state of L93A was shown to be 13-*cis* by extraction of the retinal (Delaney et al., 1995). It was suggested that interaction of the 13-methyl group with the bulky leucine side chain at position 93 is necessary for the final rapid reisomerization.

Another example for the increased accumulation of the O intermediate is in mutants of Glu-204. E204Q and E204D exhibited long-lived O-like intermediates with difference maxima near 640 nm like the wild type (Brown et al., 1995; Richter et al., 1996a,b). Their accumulation is presumably the consequence of the perturbation of the deprotonation of Asp-85 by the absence or modification of the proton acceptor at position 204. In the mutants of Glu-204 perturbations of the chromophore must be small because this residue, near the extracellular surface, is remote from the chromophore. These mutants are expected to give an opportunity to record unperturbed FTIR spectra of the O intermediate.

The present study explores the structural characteristics of the last step in the photocycle and the dynamics of the protein structural changes by means of TR-FTIR spectroscopy in the two different kinds of mutants that accumulate the red-shifted intermediates: the mutants of Glu-204 and Leu-93. The results clearly show that the chromophore structure of the red-shifted intermediate is O-like in the Glu-204 mutants and N-like in the Leu-93 mutants, the latter being consistent with the previous result (Delaney et al., 1995). However, both red-shifted intermediates have a protein structure similar to that of the wild-type O intermediate. The fact that the red-shifted intermediates of the Leu-93 mutants have N-like chromophore structure with an O-like protein structure leads to a conclusion that protein structural changes in the last step of the photocycle will proceed regardless of the chromophore structure. In addition to the structural features of the last intermediates, the kinetics showed a delay of the deprotonation of Asp-204 relative to the protonation of Asp-85 in the L-to-M transition of E204D, suggesting the occurrence of protein structural changes that lead to the delay of the proton release.

MATERIALS AND METHODS

The bacteriorhodopsin mutants E204Q and E204D, as well as L93M, L93T, L93S, L93V, L93I, L93E, and L93F, were constructed as described previously (Cao et al., 1993; Brown et al., 1995), and the protein was prepared as purple membranes by the standard method (Oesterhelt & Stoeckenius, 1974). The membrane suspensions in 1 mM phosphate buffer at pH 6.0 (E204Q and E204D) or in distilled water (L93 mutants) were dried on a BaF_2 window (18 mm diameter) by allowing evaporation overnight. We put 3 μL of water beside the dried film, sealed it with the second window, and kept it in the cryostat (DN-1704, Oxford) in the FTIR spectrometer. Measurements of the absolute infrared spectrum after the experiments assured that no loss of water occurred during recordings of 8 h. The temperature of the sample film was kept at 298 K through the experiments by a temperature controller (ITC-4, Oxford).

Time-resolved absorption spectroscopy in the visible range was done as described previously (Brown et al., 1994), with a hydrated film of the mutants of Leu-93 produced by drying membranes in water on the inner surface of a cuvette and hydrating it fully with water-soaked filter paper. The measurements were at 298 K.

Time-resolved infrared spectra of the mutants were recorded by use of a commercial step-scan FTIR instrument (FTS-60A/896, Bio-Rad), which can provide time-resolved spectra every 2.5 μs. The time resolution (instrumental response) was evaluated to be 20 μs by measuring the rise of the negative peak at 1528 cm^{-1} (C═C stretch) in the L minus BR spectrum of the wild-type. This is fast enough for the present measurement. The spectral resolution was 4 cm^{-1}, providing data points every 2 cm^{-1}. The sample was excited at 540 nm with an excimer-pumped dye laser (Lambda-Physik, EMG-101MSC and FL-3002). The excitation laser power was 1.4 mJ. The sample film was tilted 45° relative to both IR probe and excitation laser beams, which made a right angle with each other. The sample was light-adapted by laser beam before recordings.

The repetition rate of excitation was different between Glu-204 and Leu-93 mutants dependent on their photocycle lifetimes. In previous studies with E204Q as a membrane suspension at pH 7.3, the photocycle lifetime was 1 s (Brown et al., 1995). Since these conditions are disadvantageous for accumulation of FTIR data, the present studies were carried out with a film at pH 6.0, where the photocycle was complete in 0.1 s. Excitation was at 5 Hz for both E204Q and E204D. Under the present conditions, one scan takes ~15 min. By averaging 16 scans (~4 h), we collected a total of 120 spectra at intervals of 1 ms. The laser excitation was provided 10 ms after the start of scanning, so that the 11th spectrum among 120 spectra was the first spectrum just

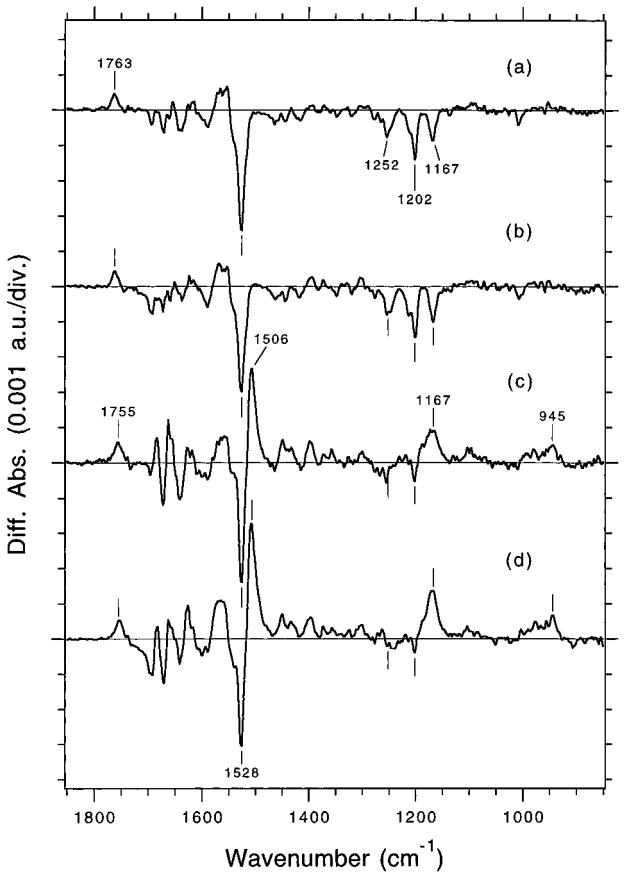

FIGURE 1: M minus BR spectra in E204Q (a) and E204D (b) and O minus BR spectra in E204Q (c) and E204D (d). The first spectra measured after excitation that contain M are shown in panels a and b, while the O minus BR spectra in panels c and d were calculated as shown in the text. The M minus BR spectra (a and b) were normalized to each other according to the negative peak at 1202 cm^{-1}. On the other hand, the O minus BR spectra (c and d) were normalized to the corresponding M minus BR spectra (a and b, respectively), according to the negative peak at 1528 cm^{-1}. Factors for expansion were 1.0, 2.8, and 3.0 for spectra b, c and d, respectively. Horizontal solid lines represent zero in the difference spectra.

after the excitation (0−1 ms).[3] The data collection was at every 1 ms after that. The average of 10 interferograms before the excitation served as the reference, and the difference IR spectra were calculated for the 120 spectra.

Excitation was at 1 Hz for L93M, where one scan takes ∼75 min. Even though some photoproducts remained at the time of the next flash for L93T and L93S, the results were the same as those at 0.25 Hz, which would have taken 300 min for one scan. By averaging seven scans, we collected a total of 120 spectra at intervals of 3 ms for L93M and 5 ms for L93T and L93S. Laser excitation was provided 30 ms after the start of scanning for L93M, and 50 ms for L93T and L93S, so that the 11th spectrum among 120 spectra was the first spectrum after photoexcitation.

RESULTS

Time-Resolved FTIR Spectroscopy of Glu-204 Mutants. The first spectrum after excitation of E204Q (Figure 1a) exhibits typical features of the M minus BR spectrum recorded at low temperature for this mutant (Brown et al., 1995): a positive band at 1763 cm^{-1} due to protonation of Asp-85, negative bands at 1528, 1252, 1202, and 1167 cm^{-1}, and the absence of positive bands in the 1200−1160 cm^{-1} region. The maximal absorbance change was −0.0034 at 1528 cm^{-1}. The M minus BR spectrum obtained for the same film (tilted by 45°) by steady illumination with >500 nm light at 230 K, which converts the BR almost completely to the M intermediate, exhibits an absorbance change of −0.025 at 1528 cm^{-1}. This indicates that 13.6% of the sample in the film was excited by each laser shot (1.4 mJ) and assures the absence of multiphoton excitation. A similar spectrum was obtained for E204D (Figure 1b).

Shortly after formation of the M intermediate, a positive band appeared at 1506 cm^{-1}, characteristic of the O intermediate of the wild type (Smith et al., 1983; Hessling et al., 1993). Then, the positive 1506-cm^{-1} band and negative 1528-cm^{-1} band decayed together. The recovery of BR, as followed by the amplitude of the negative 1528-cm^{-1} band, could be fitted with a single exponential with a time constant of 17.0 ± 0.2 ms for E204Q and 14.1 ± 0.3 ms for E204D (not shown). In the films the photocycle of E204Q was only slightly slower than that of E204D, in contrast to the previous results with membrane suspensions at pH 7.3, in which the photocycle of E204Q was an order of magnitude slower than that of E204D (Brown et al., 1995).[4] The time constants of the rise and decay components at 1506 cm^{-1} were as follows: the rise components were 8.0 ± 0.5 ms for E204Q and 6.0 ± 0.7 ms for E204D, and the decay components were 18.5 ± 1.2 ms for E204Q and 12.4 ± 1.2 ms for E204D, nearly the same as those for the recovery of BR probed at 1528 cm^{-1}. Thus, all spectral changes relaxed completely by 100 ms.

In the normal photocycle of the wild type, the N state forms after the M intermediate. With the mutants of Glu-204 at pH 6.0, the N intermediate was not detected as an isolated component, however, with a distinct time constant in singular value decomposition analysis (Henry & Hofrichter, 1992). Furthermore, an N-specific intense amide I band at 1649 cm^{-1} and an amide II band at 1557 cm^{-1}, which could be demonstrated in authentic N minus BR spectra of E204Q and E204D when recorded by static FTIR measurement at pH 10 and 275 K (not shown), were undetectable in the measurements at ambient temperature.

Spectral Features of the O Intermediates of E204Q and E204D. Figure 1 also shows the O minus BR spectra of E204Q (spectrum c) and E204D (spectrum d). Twenty spectra between 7 and 26 ms in E204Q, which contained large contributions of the O intermediate, were averaged. The O minus BR spectrum of E204Q (Figure 1c) was then obtained by subtracting the contribution of the M minus BR spectrum (∼10%), which is still present in this time range, from the averaged spectrum so as to remove the M-specific band at 1762 cm^{-1}. Similarly, the O minus BR spectrum of E204D (Figure 1d) was obtained by subtraction of the M minus BR spectrum (∼5%) from the average of 13 spectra between 11 and 23 ms.

[3] In an experiment with better time resolution, the actual delay time for the start of the 11th spectrum was estimated to be 10 ms after photoexcitation.

[4] The previous study by Brown et al. (1995) was done with the suspension in 2 M NaCl (pH 7.3). On the other hand, the present study was conducted with the hydrated film obtained from 1 mM phosphate buffer (pH 6.0). Kinetic features of this film were almost identical with those of the suspension.

The O minus BR spectra of E204Q and E204D (Figure 1c,d) resemble each other. The shift of the C═C stretch from 1528 to 1506 cm^{-1} upon formation of the O intermediate is consistent with the red shift of the absorption maximum. The negative bands at 1252, 1202, and 1167 cm^{-1} of BR are canceled and a positive band at 1167 cm^{-1} appears in both E204Q and E204D. The intense 1167-cm^{-1} band is a characteristic feature of the resonance Raman spectrum of the O intermediate which contains an *all-trans* chromophore (Smith et al., 1983). The positive band at 1755 cm^{-1} is due to protonated Asp-85. The hydrogen out-of-plane (HOOP) vibrational bands at 945 cm^{-1} and at higher frequencies are similar to the 977-, 959-, and 945-cm^{-1} bands in the resonance Raman spectrum of the O intermediate (Smith et al., 1983). This region was not shown in the previously reported O minus BR FTIR spectrum of the wild type (Hessling et al., 1993). Thus, *the chromophore structure in the O intermediates of E204Q and E204D is identical with that of the wild type*. This is probably the result of an absence of perturbation of the chromophore from remote Glu-204.

The baseline distortions, which were estimated by the same averaging without laser illumination in the amide I and carboxylic C═O stretch regions, are smaller than 1×10^{-4} absorbance unit. Both E204Q and E204D exhibited negative bands at 1695, 1672, and 1642 cm^{-1}, and positive bands at 1684, 1663, 1655, 1628, and 1618 cm^{-1} (Figure 2a) with some variations in intensities (Figure 2b). Among them, the negative band at 1642 cm^{-1} and the positive band at 1628 cm^{-1} are the C═N stretches of the protonated Schiff base of BR and the O intermediate, respectively, as judged from the resonance Raman spectra (Smith et al., 1983). The other bands probably originate from amide I, the C═O stretch of peptide carbonyl (Krimm & Bandekar, 1986).

The negative diffuse signal of E204D at around 1714 cm^{-1} (Figure 2b), which is absent in E204Q (Figure 2a), is related to the deprotonation of Asp-204 as the same negative band in the M minus BR spectrum of E204D with a peak at 1714 cm^{-1} (Brown et al., 1995). The reasons for its broad shape are not clear. The presence of the negative 1714-cm^{-1} signal of Asp-204 and the positive 1755-cm^{-1} signal of Asp-85 in the O minus BR spectrum of E204D indicates that the proton transfer from Asp-85 to Asp-204 occurs in the final O-to-BR process in the photocycle. The negative band at 1732 cm^{-1} in E204Q and E204D must originate from the C═O stretch of Asp-115 because the C═O stretch of Asp-96 is at 1743 cm^{-1} in the N minus BR spectrum (not shown). These frequencies of Asp-115 and Asp-96 are similar to those of the wild type (Sasaki et al., 1994), implying that the surroundings of these residues are not altered in the E204 mutants. Figure 2 also shows that Asp-115 changes its frequency from 1732 to 1738 cm^{-1} upon O formation. Such a weakened H-bonding of the C═O group of Asp-115 is also seen for the M and N intermediates of the wild type (Sasaki et al., 1994). On the other hand, the absence of any spectral perturbation of Asp-96 in the O intermediate indicates that the structure around Asp-96 in the cytoplasmic region is completely restored in the O intermediate, as for the wild type (Hessling et al., 1993).

Regarding the C═O stretch of the protonated Asp-85 (1763 cm^{-1} in M and 1755 cm^{-1} in O), a slight difference in frequency was noticeable between E204Q and E204D. Fitting of the spectra to a Gaussian function allowed

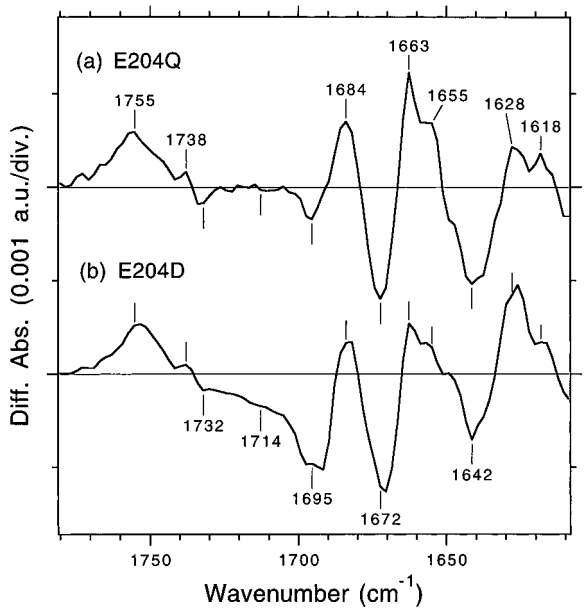

FIGURE 2: O minus BR spectra in E204Q (a) and E204D (b) in the 1780−1610 cm^{-1} region. The spectra are reproduced from Figure 1c,d. The baseline distortion is less than 1×10^{-4} absorbance unit. Horizontal solid lines represent zero in the difference spectra.

estimation of the peak position of the C═O stretch with 0.1 cm^{-1} resolution. E204Q and E204D exhibited peaks at 1763.0 and 1761.5 cm^{-1} in the M intermediate and at 1755.7 and 1753.8 cm^{-1} in the O intermediate, respectively. Similar analysis applied to the earlier published spectra at low temperature (Brown et al., 1995) showed the peak positions at 1763.3, 1761.7, and 1761.1 cm^{-1} in the M intermediates of E204Q, wild type, and E204D, respectively. These results indicate that H-bonding strength of the C═O group of Asp-85 is slightly impaired when the negative charge at position 204 is absent during the photocycle. These results are also an indication that the C═O group of Asp85 in a nonpolar environment (Dioumaev & Braiman, 1995) senses the electric field from the residue at position 204.

Time-Resolved FTIR Spectroscopy of Leu-93 Mutants and Spectral Features of the O-like Intermediates of Leu-93 Mutants. Unlike with Glu-204 mutants, TR-FTIR was difficult to do with the mutants of Leu-93 because the slow turnover of their photocycles forced us to lower the repetition rate of the recordings. For 4 cm^{-1} spectral resolution in the present spectrometer, one scan took 7.5 min at 10 Hz. We found that L93M had the shortest photocycle (overall time constant ~250 ms) among the tested mutants of Leu-93[5] and allowed one scan in 75 min at 1 Hz. According to visible transient absorption of L93M, the red-shifted intermediate appears in the several millisecond time range from a mixture of L and M states, in which the amount of M is unusually low (not shown). The difference spectrum in the visible range between the red-shifted intermediate and the unphotolyzed state had a minimum at 530 nm and a maximum at 610 nm, with an isosbestic point at 575 nm. The red-shifted photoproduct, referred to as the O intermediate in previous reports (Cao et al., 1993; Delaney et al., 1995), decayed to the initial BR state within 1 s.

According to spectra in the visible range, the first TR-FTIR spectrum just after excitation (0−3 ms) contains mostly

[5] We measured the photocycles of seven mutants of Leu93: L93M, L93T, L93S, L93V, L93I, L93E, and L93F.

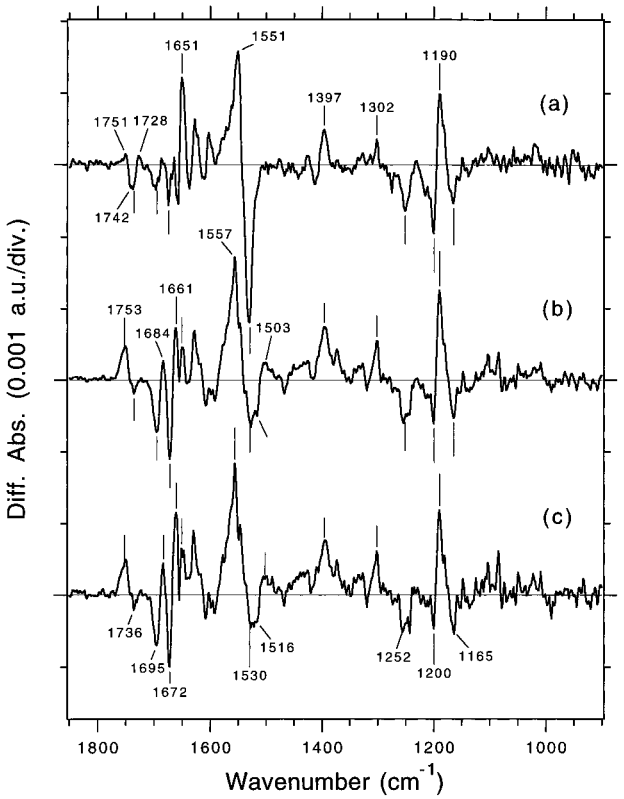

FIGURE 3: Typical infrared difference spectra of L93M. (a) First spectrum measured immediately after excitation. (b) Average of 20 spectra in the 42−102 ms domain. (c) Average of 50 spectra in the 177−327 ms domain multiplied by 1.55 so that the positive band at 1753 cm^{-1} has the same intensity as spectrum b.

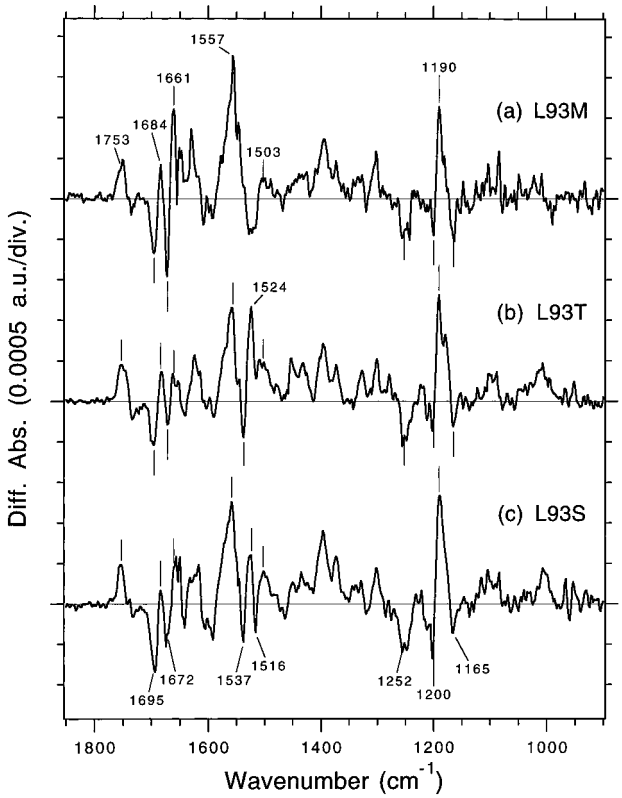

FIGURE 4: Infrared spectra of the long-lived red-shifted intermediate of L93T (b) and L93S (c) in the 100−350 ms range, compared with that of L93M (a) reproduced from Figure 3c.

the L state and small amounts of M and red-shifted intermediate. In the TR-FTIR spectra of L93M, the first spectrum (0−3 ms; Figure 3a) exhibits a negative band at 1742 cm^{-1} for Asp-96. The shift of the C═O stretch vibrations of the protonated carboxylic groups from 1742 to 1751 cm^{-1} for Asp-96 and from 1736 to 1728 cm^{-1} for Asp-115 are similar, if not identical, to those of the L minus BR spectrum of the wild type (Braiman et al., 1988; Maeda et al., 1992). The negative band of the C═C stretch at 1530 cm^{-1}, at a slightly higher frequency than the wild type (1528 cm^{-1}), is consistent with the blue-shifted visible absorption spectrum of the unphotolyzed state (at 547 nm). A more intense positive band at 1551 cm^{-1} than the wild type might be due to the C═C stretch of the L intermediate. The three negative bands at 1252, 1200, and 1165 cm^{-1}, with a positive band at 1190 cm^{-1}, are characteristic of the formation of a 13-*cis* photoproduct from *all-trans*-BR. The positive band is intermediate between the frequencies of the L and N intermediates of the wild type, at 1192 and 1186 cm^{-1}, respectively. The intense chromophore bands at 1397 and 1302 cm^{-1}, and the absence of the negative bands at 1640 and 1010 cm^{-1}, are more typical of the N minus BR spectrum of the wild type than the L minus BR spectrum.

After formation of the L intermediate, a positive band at 1753 cm^{-1} appears. The positive band of the M intermediate at 1762 cm^{-1} is barely observable throughout the entire series of the TR-FTIR spectra. The entire series of spectra obtained with the 3-ms interval, except for the first few after excitation, corresponds to the red-shifted intermediate in the visible region. The TR-FTIR spectra obtained as averages of the spectra in the 42−102 (Figure 3b) and 177−327 (Figure 3c) ms time domains were essentially identical with each other, indicating persistence of the same states after the decay of the L intermediate. There are three negative bands at 1252, 1200, and 1165 cm^{-1} as well as a positive band at 1190 cm^{-1} in the fingerprint region as in Figure 3a, though the negative band at 1200 cm^{-1} is less intense. This looks similar to the N minus BR spectrum of the wild type and is typical for the *all-trans* to 13-*cis* conversion. In other words, the red-shifted intermediate possesses a 13-*cis* chromophore and is not produced from a 13-*cis* species possibly present in light-adapted L93M. The much smaller intensity of the negative band at 1530 cm^{-1} in both spectra b and c than in spectrum a of Figure 3 could be due to cancellation by a positive C═C stretch band near 1530 cm^{-1} in this state.[6] The 1503-cm^{-1} positive band in L93M is too broad in width and weak in intensity to regard it as the C═C stretch, unlike the band at the same frequency in the mutants of Glu-204 (Figure 1). In any case, the absorption maximum of the photoproduct is not so red-shifted as to predict a C═C stretch frequency of 1503 cm^{-1}. A similar band is present in the M intermediate and it is missing when Tyr-185 is labeled (Liu et al., 1995). Thus, this band is not characteristic of the O intermediate.

These spectra are distinct from that of the O intermediate of the mutants of Glu-204 (Figure 1) with respect to the absence of the positive band at 1167 cm^{-1} and the HOOP bands in the 1000−900 cm^{-1} region. The intense bands at

[6] The invariant frequency of the C═C stretch at 1530 cm^{-1} in both the unphotolyzed and the red-shifted states with the different λ_{max} is not in conflict with a well-known linear inverse relation between the absorption maximum and the frequency of the C═C stretch (Aton et al., 1977), because the relation is just qualitative and several species show deviations from it.

1557, 1397, and 1302 cm^{-1} are characteristic of the N intermediate of the wild type. This spectrum is almost identical as regards the chromophore bands with that of L93M that was recorded under the standard conditions for the pure N minus BR spectrum of the wild type (pH 10 at 273 K, spectrum not shown) (Pfefferlé et al., 1991). However, the positive amide I bands at 1684 and 1661 cm^{-1} are common with the O intermediate of the Glu-204 mutants (Figure 2), and they are absent in the N intermediate. The negative amide I bands at 1695 and 1672 cm^{-1} are also common with the O intermediate of the Glu-204 mutants, whose frequencies are slightly lower than those of the N intermediate. The negative band at 1742 cm^{-1} due to Asp-96 is absent, also as usual in the O intermediate.

The TR-FTIR spectroscopy thus revealed that the red-shifted intermediate of L93M has a 13-*cis* chromophore, as reported earlier from extraction of the retinal (Delaney et al., 1995), and that the chromophore structure is more like that of the N intermediate. The generality of these properties was tested with L93T and L93S. Averaged spectra of L93T and L93S in the 100−350 ms time domain are shown in Figure 4, panels b and c, respectively, along with the corresponding spectrum of L93M (Figure 4a), reproduced from Figure 3c for the sake of comparison. As in the case of L93M, only *all-trans* to 13-*cis* photoreactions were observed. The same amide I bands were found, and at the same frequencies, but with some variations in intensities. Most significant of the differences is the emergence of a unique positive band at 1524 cm^{-1} in L93T and L93S. This might be due to the shift of the C═C stretch from 1530 cm^{-1} in the unphotolyzed state to 1524 cm^{-1} in the photointermediates of L93T and L93S. This would be in contrast to the unchanged frequency of 1530 cm^{-1} in the photointermediate of L93M.

Dynamics of the Protonation Changes in Asp-85 and Asp-204. As discussed above, the final step of the photocycle (the decay of O intermediate) is accompanied by proton transfer from Asp-85 to Glu-204. Although in E204Q the proton trapped at Asp-85 can be only released directly to the extracellular aqueous phase in the late step, in E204D it will be transferred to Asp-204 because this mutant releases a proton from Asp-204 earlier in the photocycle (Brown et al., 1995). The chronological relation between the protonation states of the two residues can be followed by TR-FTIR analysis of the L-to-M and O-to-BR transitions in E204D.

Figure 5 shows the time courses of the intensities at 1765 (+) (panels a and d), 1753 (+) (panels b and e), and 1714 (−) (panels c and f) cm^{-1} for E204Q (panels a−c) and E204D (panels d−f). The frequencies at 1765 and 1753 cm^{-1} are representative of the C═O stretches of Asp-85 in the M and O intermediates, respectively. A double-exponential fitting of the kinetics at 1753 cm^{-1} yielded decay time constants of 21.5 ± 1.1 ms for E204Q (Figure 5b) and 14.9 ± 0.7 ms for E204D (Figure 5e). The deprotonation of Asp-85 occurs with similar time courses as the decay of the O intermediate (18.5 ms for E204Q and 12.4 ms for E204D) and the recovery of BR (17.0 ms for E204Q and 14.1 ms for E204D). It should be noted that the apparently faster rise at 1753 cm^{-1} (Figure 5b,e) than that at 1506 cm^{-1} (8.0 ms for E204Q and 6.0 ms for E204D) probably originates from the contribution of the 1765-cm^{-1} band of the M intermediate to the intensity at 1753 cm^{-1}. It could indicate that the N intermediate accumulates transiently at this time, even if not clearly distinguished in the TR-FTIR spectra.

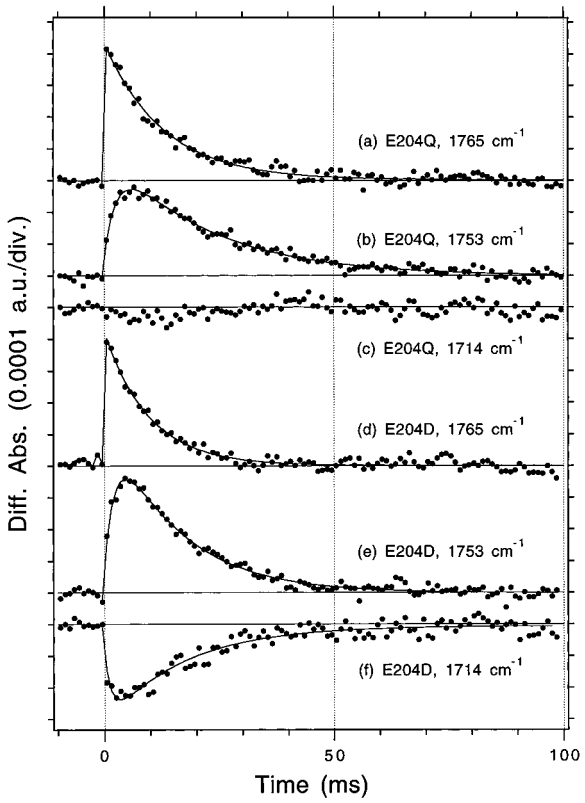

FIGURE 5: Time-dependent changes of absorbance at 1765 cm^{-1} (a and d), 1753 cm^{-1} (b and e), and 1714 cm^{-1} (c and f) in E204Q (a−c) and E204D (d−f). Smooth lines represent fits, where single- or double-exponential fitting was applied for panels a and d or for panels b, e, and f, respectively. Horizontal solid lines represent zero difference absorbance.

The negative intensity at 1714 cm^{-1} (Figure 5f) is related to the deprotonation of Asp-204 because it is absent in E204Q (Figure 5c), as reported previously for the M minus BR spectra (Brown et al., 1995). Comparison of the formation kinetics in Figure 5, panels d and f, yielded an interesting result. We had shown that the M minus BR difference spectrum measured at 230 K exhibits both positive 1765-cm^{-1} and negative 1714-cm^{-1} bands, indicating that Asp-85 is protonated and Asp-204 is deprotonated in the M intermediate (Brown et al., 1995). Nevertheless, the kinetics of E204D in the 0−10 ms time domain are different at 1765 cm^{-1} (Figure 5d) and 1714 cm^{-1} (Figure 5f). At 1765 cm^{-1}, the first measured time point after excitation has maximal amplitude already and then decays monotonously (Figure 5d), while at 1714 cm^{-1} there is a lag period in the negative signal (Figure 5f).[7] *This result suggests that the deprotonation of Asp-204 is delayed relative to the protonation of Asp-85.*

The negative signal at 1714 cm^{-1} decayed with a time constant of 16.7 ± 1.6 ms. This is coincident with the deprotonation of Asp-85 (1753 cm^{-1}; 14.9 ± 0.7 ms). Thus, *the proton transfer from Asp-85 to Asp-204 at the end of the photocycle of E204D occurs without an intervening intermediate state.*

[7] This was observed for the E204D film not only at pH 6.0 but also at pH 7.0 and 8.0. On the other hand, more recent experiments with 5 μs time resolution have shown that part of the negative band is already present before protonationof Asp-85.

DISCUSSION

Chromophore Structure of the Red-Shifted Photointermediates of the Mutants of Glu-204 and Leu-93. The present TR-FTIR measurements clearly show a difference in the chromophore structures of the last, red-shifted intermediates of the photocycles of Glu-204 and Leu-93 mutants. The O state of E204Q and E204D shows three HOOP bands at 945 cm^{-1} and higher frequencies, the intense C−C stretch band at 1167 cm^{-1}, and the sharp C═C stretch band at 1506 cm^{-1} (Figure 1) like the O intermediate of the wild type in the resonance Raman spectrum (Smith et al., 1983). These bands were shown to arise from the twisted *all-trans* chromophore with a difference absorption maximum at 640 nm. We thus conclude that the chromophore structure of the mutants of Glu-204 is identical to that of the wild type.

The previously measured FTIR spectrum of the O intermediate of Y185F (Bousché et al., 1992) also exhibited the same C═C and C−C stretch bands but with only a single band at 956 cm^{-1} in the HOOP region. Thus, the HOOP modes in the O intermediate are affected by substitution of Tyr-185. The O minus BR spectrum of the wild type by Hessling et al. (1993) showed a similar feature to those, though with some differences in frequency. The data by Smith et al. (1983), though not explicitly described by themselves, shows that the 974-cm^{-1} band is due to the combination band of C_{15} and N HOOP. The difference of 39 cm^{-1} between the C═NH stretching at 1628 cm^{-1} and C═ND stretching at 1589 cm^{-1} indicates quite strong H-bonding of the Schiff base (Rodman-Gilson et al., 1988) and an unusual structure of the C═N bond. The lack of the 974-cm^{-1} band and a long lifetime of the O intermediate in Y185F suggest the interaction of the Schiff base with Tyr-185, probably through a residue connected to it, such as Asp-212, in the O intermediate.

In contrast to the O intermediate of the Glu-204 mutants, the red-shifted long-lived intermediate of L93M displays none of the HOOP bands. Persistence of both the negative band at 1165 cm^{-1} and positive band at 1190 cm^{-1} from the N-like state suggests that the chromophore of the red-shifted intermediate is in the 13-*cis* form. The intense bands at 1397 and 1302 cm^{-1} are also characteristic of the N intermediate (Pfefferlé et al., 1991). Thus, from the point of view of the chromophore structure, the red-shifted intermediate of L93M is not an O state but an N state. This occurs presumably because of a defect in the specific interaction between Leu-93 and retinal skeleton, as pointed out by Delaney et al. (1995).

Protein Structure of the Red-Shifted Photointermediates of the Mutants of Glu-204 and Leu-93. The TR-FTIR measurements reveal also protein structural changes in these intermediates, mainly by probing the amide I region. One of the characteristics of the N state is the intense bands of amide I at 1692 (−), 1670 (−), and 1650 (+) cm^{-1} (Pfefferlé et al., 1991; Sasaki et al., 1992; Hessling et al., 1993; Rothschild et al., 1993). The spectrum of the O intermediate of E204Q and E204D contained the two negative bands, although with slightly higher frequencies at 1695 and 1672 cm^{-1}. The positive band was replaced by bands at 1684, 1663, and 1655 cm^{-1}.

The frequency of the C═O stretch of Asp-115 shifts from 1732 to 1738 cm^{-1} in the O intermediates of E204Q and E204D. It is known that this frequency is down-shifted in the K and L intermediates, while it is up-shifted in the M and N intermediates of the wild type (Braiman et al., 1988; Sasaki et al., 1994). Thus, the H-bonding of the C═O group of Asp-115 is first strengthened upon photoisomerization and then weakened in the L-to-M transition, an effect that persists in the O intermediate. On the other hand, no spectral change of Asp-96 was observed in the O minus BR spectra of E204Q and E204D, as in the wild type (Hessling et al., 1993) and Y185F (Bousché et al., 1992). Since the N intermediate under these conditions contains deprotonated Asp-96, this fact implies that the N-to-O transition is accompanied not only by protonation of Asp-96 from cytoplasmic aqueous phase but also by the complete restoration of the environment around Asp-96. Thus, the protein structure at the cytoplasmic side seems to be restored in the O intermediate.

In the red-shifted species of L93M some aspects of the protein are characteristic of the O state, even though the chromophore (and surrounding protein) structure is still that of an N state. Indeed it exhibits positive amide bands at 1661 and 1684 cm^{-1}, as were observed for the O intermediate of the Glu-204 mutants (Figure 4). In addition to the peptide backbone, the absence of the negative band at 1742 cm^{-1} of an unprotonated Asp-96 is usually also characteristic of the O intermediate. The spectrum further shows the absence of any perturbation around Asp-96 in this state, indicating complete restoration of the environment of Asp-96 as normally occurs in the O intermediate. These facts indicate that the protein structural changes proceed from the N state to the O state even though the chromophore structure is still at the N state. A similar protein change in advance of the chromophore change was reported in the M-to-N transition of a mutant D96N by finding an intermediate state possessing the M-like chromophore structure (unprotonated Schiff base) and the N-like protein structure (prominent amide I band), which was called M_N (Sasaki et al., 1992). That study revealed that the protein structural changes in the M-to-N transition proceed regardless of the protonation state of the chromophore. The present study shows that the protein structural changes in the N-to-O transition also proceed without regard to the isomeric state of the chromophore.

Structural Changes of the Asp-85−Glu-204 Region in the L-to-M and O-to-BR Transitions. Interaction between Asp-85 and Glu-204 causes the deprotonation of Glu-204 upon protonation of Asp-85 in the L-to-M transition. The interdependency of the pK_as of these residues in the unphotolyzed state could be demonstrated upon mutations of these residues (Richter et al., 1996a). The interaction at the long distance (∼1 nm) that separates Asp-85 and Glu-204 (Henderson et al., 1990; Grigorieff et al., 1996) can be achieved electrostatically (Richter et al., 1996a) including the interaction with the intervening Arg-82 (Balashov et al., 1993; Govindjee et al., 1996). It might be expected that such electrostatic interactions would occur rapidly and cause the virtually simultaneous protonation of Asp-85 and deprotonation of Glu-204. The delay in the proton migration to a covalently linked fluorescein dye just close to the surface relative to the L-to-M transition (Cao et al., 1995) suggested, however, that the deprotonation of Glu-204 is not necessarily concurrent with protonation of Asp-85. The observation of a delay in the deprotonation of Asp-204 after protonation of Asp-85 (Figure 5) indicates more explicitly that the coupling between the two residues is not rapid in the case of E204D.

The slow response of Asp-204 to the protonation of Asp-85 suggests that the change in the pK_a of the proton release group requires structural changes of the protein in the extracellular side, at least in E204D. This region was suggested to contain a H-bonding network that includes water molecules (Humphrey et al., 1994). Substitutions of Glu-204 and Arg-82 will influence the O−H stretch of the water molecule coordinated to Asp-85. The water molecules between Arg-82 and Glu-204 are affected by the H-bonding ability of these residues (Brown et al., 1995; Hatanaka et al., 1996). The C═O stretches of some peptide bonds and Asp-85 in the M and O intermediates are influenced by substitution of Glu-204 (present study). Orientation change of Arg-82, as suggested to occur in the M intermediate, may be involved in this process (Bashford & Gerwert, 1992; Balashov et al., 1993; Scharnagl et al., 1995; Hatanaka et al., 1996).

On the other hand, at the end of the photocycle the protonation of Asp-204 and the deprotonation of Asp-85 in the O-to-BR step proceed with similar time constants (Figure 5), indicating rapid proton movement between the two residues. Some peptide carbonyls (Figure 2) may mediate it. Water molecules may also participate in the long-range proton transfer. Direct observation of the O−H stretch region of the O minus BR spectrum will provide the mechanism of the water-mediated proton transfer, as it did earlier for the proton transfer between the Schiff base and Asp-85 (Maeda et al., 1994; Kandori et al., 1995). This will be our future focus.

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