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Structural Changes in the Peptide Backbone in Complex Formation between Activated Rhodopsin and Transducin Studied by FTIR Spectroscopy†

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ABSTRACT: Structural changes in the complex formation between transducin and metarhodopsin II, the activated form of photolyzed rhodopsin, in visual transduction processes were analyzed by Fourier transform infrared spectroscopy. The spectrum of the complex was obtained by subtracting the contribution of metarhodopsin I and uncomplexed metarhodopsin II. The averaged spectrum upon the complex formation was then compared with that in the conversion of rhodopsin-to-metarhodopsin II. Frequency shifts of the peptide carbonyl vibrations at 1686, 1674, and 1661 cm^{-1} to 1640 cm^{-1} were observed upon complex formation from metarhodopsin II plus transducin. These changes must have resulted from the strengthening of H-bonding of one or a few peptide groups but is not ascribable to global conformation change. Changes in the frequencies of the peptide amides were also detected. With regard to intramembrane carboxylic acid residues, no further changes were noticed in the carboxyl vibrations of Asp83, Glu122, and Glu113. Only a small change possibly due to Glu134 was detected.

Receptor proteins with transmembrane heptahelical structure and heterotrimeric G proteins are widely involved in cellular signal transducing systems (Kaziro et al., 1991; Neer, 1995). Among them, a rhodopsin−transducin system is best in studying the signal transduction mechanism at molecular level (Hofmann, 1986). The active form of photolyzed rhodopsin is metarhodopsin II (MII).[1] It has an unprotonated Schiff base and is formed in equilibrium with its precursor, metarhodopsin I (MI), with a protonated Schiff base (Mathews et al., 1963; Imai et al., 1994). The GDP-bound form of transducin increases MII, indicating complex formation between MII and transducin (Emeis & Hofmann, 1981). The α-subunit of transducin (Tα) in the complex releases the bound GDP from the binding site, and GTP fills the now-empty site (Bornancin et al., 1989). Tα then dissociates from the complex, and the original equilibrium mixture of MI and MII is restored. Released Tα then activates cyclic-GMP phosphodiesterase and eventually leads to electrical potential changes at the plasma membrane (Stryer, 1991).

The binding sites in the interaction between rhodopsin and transducin were identified in competition experiments by use of synthetic peptides of the cytoplasmic loops of rhodopsin (König et al., 1989) or of the C-terminus domains of Tα (Hamm et al., 1988). The deletion mutagenesis of rhodopsin have also shown the cytoplasmic loops as the sites for the

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[1] Abbreviations: MII, metarhodopsin II; MI, metarhodopsin I; Tα, α-subunit of transducin; FTIR, Fourier transform infrared; HOOP, hydrogen out-of plane.

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interaction (Franke et al., 1990, 1992). A close contact between one of the loops of rhodopsin and Tα was shown by photoaffinity labeling (Resek et al., 1994). The importance of hydrophobic amino acid residues in the C-terminus region of transducin was shown also by mutational studies (Garcia et al., 1995; Osawa & Weiss, 1995; Martin et al., 1996). X-ray crystallographic analysis on the structure of transducin (Lambright et al., 1994, 1996), however, has not revealed further the modes of the interaction between transducin and MII. An NMR study detected conformational changes in the synthetic peptide composed of the 11-mer of the C-terminus of Tα upon interaction with MII (Dratz et al., 1993).

Difference Fourier transform infrared (FTIR) spectroscopy is capable of detecting changes in chemical bonds of proteins (Maeda, 1996) and has been used to detect changes in the photochemical intermediates of rhodopsin for the intramembrane carboxylic acid residues (Fahmy et al., 1993; Rath et al., 1993; Jäger et al., 1994; DeCaluwé et al., 1995; Nishimura et al., 1995), peptide bonds (Ganter et al., 1988; Nishimura et al., 1995), and internal water molecules (Maeda et al., 1993; Kandori & Maeda, 1995). The present paper applied this technique to the complex between MII and transducin and examined structural changes upon complex formation.

MATERIALS AND METHODS

Rhodopsin in bovine rod outer segments was prepared as described previously (Kandori & Maeda, 1995). Transducin was extracted from it and purified by chromatography on a DEAE Toyopearl column (10 × 60 mm) (Fukada et al., 1994). After GTP was removed by washing the column with 0.1 M NaCl in 10 mM MOPS buffer, pH 7.5, containing 2 mM $MgCl_2$, 1 mM DTT, and protease inhibitors, the trimeric transducin was eluted with 0.6 M NaCl and then dialyzed against 0.1 M NaCl in the same buffer. Subsequent procedures were done under dim red light. The rhodopsin (6 μM) and transducin (12 μM) were mixed and precipitated together by centrifugation at 90 000 rpm for 40 min (in a TLA100.3 rotor, Beckman Ultracentrifuge model TL100). About 60% of the added transducin was precipitated with rhodopsin as judged by densitometric scanning of a gel in which the supernatant was electrophoresed (not shown). The final concentrations of rhodopsin and transducin were estimated to be 2.3 and 2.8 mM, respectively. About 0.5 μL of the pellet was scraped by a spatula and transferred to a BaF_2 window. The concentrated mixture was sandwiched by another BaF_2 window with a Teflon spacer of 12.5 μm thickness. For the pellet with GTPγS, an unhydrolyzable analog of GTP, 30 mM GTPγS was added to the mixture of rhodopsin and transducin before the centrifugation. The final molar ratio of GTPγS to transducin in the pellet was about 5.

Difference FTIR and visible spectra upon illumination were obtained as reported previously (Nishimura et al., 1995). Each FTIR spectrum was depicted from 32 interferograms with 2-cm^{-1} resolution and was sequentially recorded at 40-s intervals. The spectra were corrected for the shape of the base line with the corresponding time-dependent spectra in the dark.

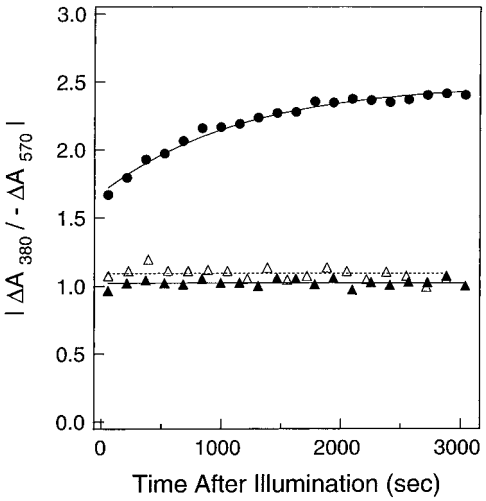

FIGURE 1: Formation of the MII−transducin complex. Changes in the total amounts of MII, which were expressed by an increase of A_{380nm} relative to a decrease in A_{570nm} due to photolyzed rhodopsin, were followed after illumination with >500 nm light for 10 s at 265 K in the absence of transducin (closed triangles), in the presence of transducin (closed circles), and in the presence of both transducin and GTPγS (open triangles). About 90% of rhodopsin underwent photoreaction.

RESULTS

The sample was illuminated with >500 nm light for 10 s at 265 K, the lowest temperature for the unfrozen pellet. Formation of MII after illumination was monitored by the increase of A_{380nm} relative to the decrease in A_{570nm}[2] due to photolyzed rhodopsin. In the absence of transducin (Figure 1; closed triangles), MII increased rapidly and then remained at a constant level up to 50 min. The addition of transducin increased the fraction of rapidly formed MII (closed circles) and further induced a second slower increase of MII that could be fitted with a single-exponential process (solid line; $\tau_{1/e} = 1180$ s). These two effects of transducin were completely abolished upon addition of GTPγS (open triangles). These confirm formation of the MII−transducin complex in the present pellet sample. The rate of the complex formation of MII is a slower process than the formation of uncomplexed MII (Fahmy & Sakmar, 1993).

The difference FTIR spectrum was calculated from the spectra before and after the illumination and was scaled for the different samples by the amount of photolyzed rhodopsin estimated from the intensity of a negative band at 970 cm^{-1} of the hydrogen out-of plane (HOOP) band in the unphotolyzed state (Figure 2). The first spectrum was recorded just after the illumination, and the following spectra were then recorded at 40-s intervals. Recordings were repeated with four, eight, and eight unilluminated new samples for those in the absence of transducin, in the presence of transducin, and in the presence of both transducin and GTPγS, respectively. The same series of recordings in the presence of transducin was repeated once more.

The photoproducts in the absence of transducin under these conditions were MI and MII (Maeda et al., 1993). The difference spectrum just after the illumination (Figure 2A, dotted line) was identical with the averaged spectrum recorded up to 600 s at 265 K (Figure 2A, solid line), indicating instantaneous equilibration between MI and MII

[2] A_{570nm} was used to avoid the contribution of MI.

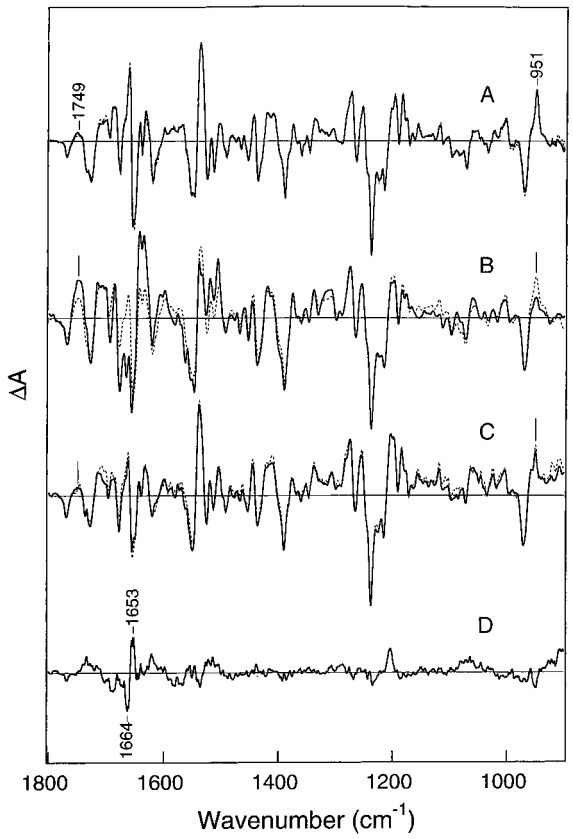

FIGURE 2: Difference FTIR spectra calculated from the spectra recorded before and after the illumination in the absence of transducin (A), in the presence of transducin (B), and in the presence of both transducin and GTPγS (C). The difference spectrum for the binding of GTPγS to transducin (C minus A) is shown in D. The samples were illuminated under the same conditions as Figure 1. The difference spectra just after the illumination are depicted by dotted lines, and those of averaged spectra up to 600 s (A and C) and at 1350 s (B) are depicted by solid lines. Dotted lines in A and C are almost invisible due to overlapping. The spectra are the average of recordings with four, eight, and eight new samples for A, B, and C, respectively. One division of the ordinate corresponds to 0.002 absorbance unit.

upon MII formation. The 951- and 1750-cm^{-1} bands in the photoproducts are characteristic of MI and MII, respectively. The content of MI in the photoproduct was estimated to be 80% from the ratio of intensity of the 951-cm^{-1} band relative to that of authentic MI measured at 240 K (Maeda et al., 1993). In the presence of transducin, the difference spectrum just after the illumination showed a greater intensity of the 1750-cm^{-1} band of MII (Figure 2B, dotted line) than in its absence (Figure 2A, dotted line). The 1750-cm^{-1} band further increased in the dark with concomitant decrease in intensity of the 951-cm^{-1} band of MI, as shown for the spectrum recorded at 1360 s after the illumination (Figure 2B, solid line). This suggests that the complex forms at the expense of the equilibrium mixture of MI and MII and does not exhibit the 951-cm^{-1} band just like uncomplexed MII. The addition of GTPγS abolished the effect of transducin on the 1750-cm^{-1} band of MII throughout the whole time range (Figure 2C), indicating that changes in the FTIR spectra induced by transducin (Figure 2B) are due to the complex formation. It also indicates instantaneous equilibration of MI and MII upon the dissociation of the complex. The subtraction of the difference spectrum without transducin from that with GTPγS plus transducin (Figure 2D, C minus A) shows a shift of an amide I band at 1664 cm^{-1} to 1653 cm^{-1}. This reflects stronger H-bonding of the peptide carbonyl upon GTP binding. This could be reconciled with structural changes in the GTP form of the Tα relative to its GDP form, as indicated by the X-ray analysis (Lamright et al., 1994, 1996).

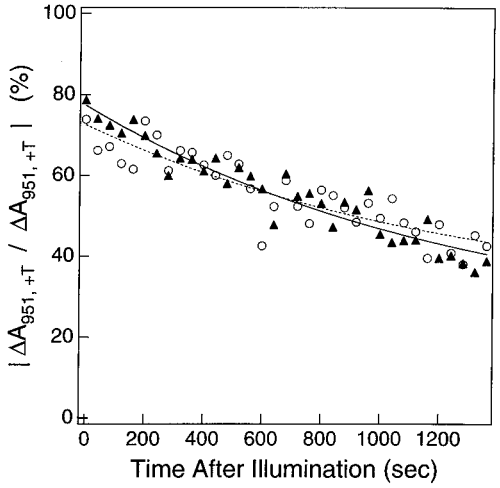

FIGURE 3: Time dependent change in the relative content of MI. Change in the ratio (%) of band intensity at 951 cm^{-1} in the presence of transducin relative to that in the absence of transducin was plotted against the duration after the offset of the illumination. The points of the first set (filled triangles) and the second set (open circles) were fitted to single-exponential curves of solid and dotted lines, respectively.

The ratio between MI and uncomplexed MII in the presence of transducin must be the same as that in the absence of transducin because there is instantaneous equilibration between MI and uncomplexed MII, as stated above. Hence, the relative content of MI plus uncomplexed MII in the presence of transducin can be deduced from the ratio of the intensity of the 951-cm^{-1} band of MI relative to that in the absence of transducin by scaling the negative band at 970 cm^{-1} as the extent of the photoreactions. The time-dependent change in the ratio of these intensities of the 951-cm^{-1} band was fitted to a single-exponential curve with $\tau_{1/e}$ of 1260 s (solid line in Figure 3) for the first set of the spectra of eight samples. This value is virtually identical with that of 1180 s estimated from the formation of MII in terms of increase in A_{380nm} (see Figure 1). This also assures that the complex formation leaves the same equilibrium mixture of MI and uncomplexed MII. A similar curve was depicted also for the second set of eight samples (dotted line in Figure 3). For each sample with 35 time-dependent spectra recorded in the presence of transducin up to 1360 s, the relative content of MI, or of MI plus uncomplexed MII, can be estimated from the intensities of the 951-cm^{-1} band. Some fluctuations of both the base line and band height at 951 cm^{-1} in the raw spectrum were eliminated by using the value on the fitted line for each time-dependent spectrum.

The difference spectrum of [rhodopsin *plus* transducin → complex] was then deduced for each time point by subtracting the averaged spectrum in the absence of transducin (Figure 2A) multiplied by the relative content of MI obtained as above. The spectra of the first set were almost identical in shape with each other. Thus, the averaged difference spectrum of [rhodopsin *plus* transducin → complex] was calculated (Figure 4A, solid line). The spectrum for the complex formation must then be compared with the difference spectrum of [rhodopsin → uncomplexed MII] at pH

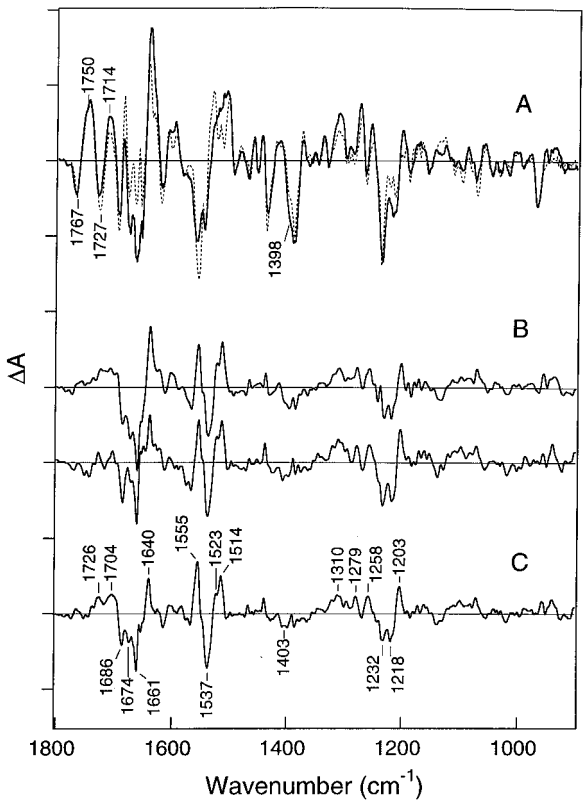

FIGURE 4: Difference FTIR spectra for the transition from MII to the complex. The spectrum of [uncomplexed MII → complex] for the first set (B, upper spectrum) was calculated by subtracting the spectrum of [rhodopsin → uncomplexed MII] (A, dotted line) from [rhodopsin *plus* transducin → complex] (A, solid line). The spectrum for the second set is shown in duplicate in B (lower spectrum), and the spectrum calculated in the same way with all 16 samples is also shown in C. One division of the ordinate corresponds to 0.001 absorbance unit.

7.5. A pure spectrum for MII formation was recorded at 285 K, where the illumination for rhodopsin did not show a band of MI at 951 cm^{-1}. This spectrum was practically free from the decay products of MII, metarhodopsin III (MIII), and opsin-plus-retinal (Van Breugel et al., 1979) because our spectrum is completely identical in shape with the [rhodopsin → uncomplexed MII] spectrum at 270 K for rhodospin at pH 5.5 (Ganter et al., 1989), where MII is more favorable than MIII (Chabre & Breton, 1979), and that obtained at pH 7 and 8 (Klinger & Braiman, 1992; Fahmy et al., 1994), where our spectrum was recorded. Furthermore, the same spectral shape remained unchanged in 37 s for the recording (Figure 4A, dotted line). Subtraction of the difference spectrum of [rhodopsin → uncomplexed MII] (Figure 4A, dotted line) from that of [rhodopsin *plus* transducin → complex] (Figure 4A, solid line) yields the spectrum for the transition of [uncomplexed MII *plus* transducin → complex] (upper spectrum in Figure 4B). The spectrum obtained in duplicate by the same procedure for second set (lower spectrum in Figure 4B) assures nice reproducibility of the frequencies in spite of different intensities for the bands at 1674 cm^{-1} and some others. The spectrum with all 16 samples was further obtained with similar procedures and presented in Figure 4C.

MII (dotted line in Figure 4A) shows the 1750(+)- and 1767(−)-cm^{-1} bands of an intramembrane carboxylic acid, Asp83 (Fahmy et al., 1993; Rath et al., 1993). They were not further affected by complex formation with transducin (Figure 4A, solid line). The perturbation of another carboxylic acid, Glu122, which was observed as the negative band at 1734 cm^{-1} in MII only in the detergents (Fahmy et al., 1993; DeCaluwé et al., 1995) and in MI (Ganter et al., 1989), did not appear either. A negative band at 1727 cm^{-1} of MII (Figure 4A, dotted line), which exhibited a slight decrease in intensity in the complex (Figure 4A, solid line), has been tentatively assigned to the peptide carbonyl close to Glu122 (DeCaluwé et al., 1995; Ganter et al., 1988). The positive band at 1714 cm^{-1} in MII (Figure 4A, dotted line) was assigned to the protonation of Glu113 (Jäger et al., 1994). A slight shift observed in the complex as a difference band at 1704 cm^{-1} (Figure 4C) does not originate from its deprotonation because of persistence of the corresponding negative band at 1398 cm^{-1} (Figure 4A, solid line vs dotted line) due to a symmetric carbonyl stretching vibration of the deprotonated Glu113 (Jäger et al., 1994).

Amide I bands, which are mainly attributed to the stretching vibration of peptide carbonyl (Krimm & Bandekar, 1986), shift from 1686, 1674, and 1661 cm^{-1} to 1640 cm^{-1} in the complex formation from MII and transducin (Figure 4C). The 1686-cm^{-1} band of MII was abolished in G90D (Zvyaga et al., 1996). They differ from the C═N stretching vibration of the unprotonated Schiff base around 1625 cm^{-1} (Siebert et al., 1983). The negative band at 1537 cm^{-1} and the three positive bands at 1555, 1523, and 1514 cm^{-1} could be due to amide II but not the C═C stretching vibration of MII because it is expected to be located at higher frequencies than those bands. Weaker positive bands at 1310, 1279, 1258, and 1203 cm^{-1} and negative bands at 1232 and 1218 cm^{-1} may not be due to the vibrations of the chromophore because these intensities must be very weak in MII with the unprotonated Schiff base. These are probably due to amide III, which are mainly attributed to the N−H bending vibrations of peptide amide (Krimm & Bandekar, 1986). Changes in amide III was shown in the photoreaction to the L and M intermediates of bacteriorhodopsin (Takei et al., 1994).

DISCUSSION

Formation of the complex does not cause further changes in the intramembrane carboxylic acid residues, Asp83, Glu122, and Glu113, which are perturbed already in MII (Fahmy et al., 1993; Rath et al., 1993; Jäger et al., 1994; DeCaluwé et al., 1995). The slight difference band at 1704 cm^{-1} (Figure 4C) might originate from the protonation of Glu134 located close to the cytoplasmic surface. It is suggested to be a site to mediate proton uptake in MII (Fahmy & Sakmar, 1993; Arnis et al., 1994). The slight depletion at 1403 cm^{-1} (Figure 4C), which are present in two spectra in (Figure 4B), can be regarded as a corresponding symmetric carbonyl stretching vibration of carboxylate. The intensities of these bands, however, were much smaller relative to others and could have been caused by partial protonation.

Changes observed for the peptide bonds can be attributed to either the transducin or the rhodopsin. The C-terminal 11 residues of Tα showed stronger interaction with MII than the Tyr316-Thr323 region of Tα (Hamm et al., 1988). Structural distortion of the peptide amides has also been proposed to occur in the C-terminal part of Tα upon complex formation (Dratz et al., 1993). The peptide bonds in the

cytoplasmic loops and the C-terminal 14 residues of rhodopsin (König et al., 1989; Franke et al., 1990, 1992; Phillips & Cerione, 1994) could also be likely candidates. Also, the site for the interaction with GDP may be included because it releases upon complex formation (Bornancin et al., 1989).

The extent of the changes observed in the peptide backbone upon complex formation from MII and transducin is less than 1 milliabsorbance unit (Figure 4C), which corresponds to a change of either a single or only a few peptide bonds for each band, when compared with the whole intensity of amide I and amide II bands in the absolute spectrum (~0.5) of the total 1100 peptide bonds. Such small changes in the peptide carbonyl are not ascribable to global conformational change. Shifts toward lower frequencies of amide I bands reflect the strengthening in H-bonding.

Involvement of the peptide carbonyl in H-bonding with a functionally important water molecule has been shown for Val49 of bacteriorhodopsin (Yamazaki et al., 1996). An FTIR study of ligand binding to a nicotinic acetylcholine receptor has revealed a peptide backbone change of a few amino acid residues (Baenziger, 1993). H-bondings of the peptide backbone are responsible for interaction in human class II histocompatibility protein with an influenza virus peptide (Stern et al., 1994). It has been shown that hydrophobic residues at positions of 344 and 349 of Tα are required for the interaction with MII (Garcia et al., 1995; Martin et al., 1996; Osawa & Weiss, 1996). Hydrophobic environments created by these residues in the interaction site of the complex would be required for the stabilization of H-bonding of the peptide carbonyls to their specific partners. It is now required that these amide bands be assigned by use of synthetic peptides of transducin and mutants of rhodopsin.

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