Transducin-Dependent Protonation of Glutamic Acid 134 in Rhodopsin†

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Received April 21, 2000; Revised Manuscript Received June 26, 2000

ABSTRACT: A highly conserved carboxylic acid residue in rhodopsin, Glu^{134}, modulates transducin (G_t) interaction. It has been postulated that Glu^{134} becomes protonated upon receptor activation. We studied the interaction between rhodopsin and G_t using Fourier transform infrared (FTIR) difference spectroscopy combined with attenuated total reflection (ATR). Formation of the complex between G_t and photoactivated rhodopsin reconstituted into phosphatidylcholine vesicles caused prominent infrared absorption increases at 1641, 1550, and 1517 cm^{-1}. The rhodopsin mutant E134Q was also studied. When measured in the presence of G_t, replacement of Glu^{134} by glutamine abolished the low-frequency part of a broad absorption band at 1735 cm^{-1} that is normally superimposed on the light-induced absorption changes of Asp^{83} and Glu^{122} of rhodopsin. In addition, a negative absorption band at 1400 cm^{-1} that is evoked by interaction of native metarhodopsin II (MII) with G_t was not observed in the difference spectrum of the E134Q mutant. Thus, Glu^{134} is ionized in the dark and exhibits a symmetrical COO^- stretching vibration at 1400 cm^{-1}. Glu^{134} becomes protonated in the G_t−MII complex and displays a C═O stretching mode near 1730 cm^{-1}. The E134Q mutation also affects absorption changes attributable to lipids, suggesting that the protonation of Glu^{134} is linked to transfer of the carboxylic acid side chain from a polar to a nonpolar environment by becoming exposed to the lipid phase when G_t binds. These results show directly that Glu^{134} becomes protonated in MII upon G_t binding and suggest that changes in receptor conformation affect lipid−protein interactions.

The large family of class I or rhodopsin-like G protein-coupled receptors (GPCRs)[1] includes visual opsins, biogenic amine receptors, neuropeptide receptors, and some chemokine receptors. The location of the ligand-binding domain in the seven-transmembrane (TM) helical bundle and the mechanism of agonist-induced receptor activation are probably conserved for all GPCRs within the rhodopsin-like class (*1, 2*). Class I GPCRs share an Asp(Glu)ArgTyr(Phe) sequence at the cytoplasmic end of TM helix 3, which moves during metarhodopsin II (MII) formation (*3, 4*). Amino acid replacements of Glu^{134} and Arg^{135} affect G_t activation (*5*−*8*), and Glu^{134} plays a central role in modulating proton uptake by MII (*9*), suggesting that its protonation may be a critical event in G_t binding or activation. The protonation states of internal carboxylic acid groups in rhodopsin have been studied by Fourier transform infrared (FTIR) difference spectroscopy in combination with site-directed mutagenesis (*10*−*12*). However, the postulated specific protonation of Glu^{134} has not been demonstrated directly in previous FTIR studies or kinetic studies of recombinant rhodopsin (*9, 13*). In addition, tentative assignments of infrared absorption bands to Glu^{134} in MII (*15*) and in MII−G_t (*14*) are conflicting.

A recent modification of the FTIR technique employed dialysis-coupled attenuated total reflection (ATR) to allow spectra of adsorbed membrane samples to be recorded under near-physiological conditions. Infrared absorption changes were recorded to probe the formation of the activated MII−G_t complex (*16*). These initial ATR-FTIR spectra suggested that a carboxylic acid group of rhodopsin apart from the chromophore-binding pocket became protonated in the MII−G_t complex.

We employed the ATR-FTIR technique to study a site-directed mutant of rhodopsin in which Glu^{134} was replaced by glutamine (E134Q). By comparing the spectra of the mutant and wild-type rhodopsin, we can assign an IR difference band to the protonation of Glu^{134} in the MII−G_t complex. Glu^{134} is ionized in the dark and exhibits a symmetrical COO^- stretching vibration at 1400 cm^{-1}. Glu^{134} becomes protonated in the G_t−MII complex and displays a C═O stretching mode near 1730 cm^{-1}. The data provide direct experimental evidence for the protonation of Glu^{134} in rhodopsin. The results also suggest that changes in receptor conformation affect lipid−protein interactions since the E134Q mutation affects absorption changes attributable to lipids. Protonation of Glu^{134} may be linked to transfer of its carboxylic acid side chain from a polar to a nonpolar environment upon G_t binding.

† This research was supported in part by the Allene Reuss Memorial Trust, Deutsche Forschungsgemeinschaft (Grant SI 278/16-1,2), and Fonds der Chemischen Industrie.

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[1] Abbreviations: DTT, dithiothreitol; GPCR, G protein-coupled receptor; G_t, transducin; IRE, internal reflection element; MI, metarhodopsin I; MII, metarhodopsin II; TM, transmembrane.

10.1021/bi000912d CCC: $19.00 © 2000 American Chemical Society
Published on Web 08/04/2000

EXPERIMENTAL PROCEDURES

Preparation of Washed Membranes and G_t. Preparation of washed membranes from bovine rod outer segments was carried out as described (*17*) with minor modifications. G_t was purified from illuminated, osmotically shocked rod outer segments by successive washes and hexyl agarose chromatography (*18*). G_t was eluted with 300 mM NaCl in buffer solution (10 mM sodium phosphate, pH 7.1, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride). The pooled peak fractions were diluted with the same buffer to a final concentration of 200 mM NaCl. The G_t final concentration was typically 10−20 μM.

Purification and Reconstitution of Recombinant Rhodopsin. The E134Q mutant was constructed in a synthetic gene (*19*) as previously described (*20*) and expressed in COS-1 cells, regenerated with 11-*cis*-retinal, and purified by immunoaffinity binding (*21*) in 1.5% *n*-octyl-β-D-glucoside (Anatrace, Inc.). Reconstitution of the recombinant pigment into lipid vesicles of L-α-lecithin from fresh egg yolk (Fluka) was done by dialysis and centrifugation as reported (*16*, *22*).

FTIR Spectroscopy. Details of the ATR measurements have been reported (*16*). In short, 1−2 nmol of recombinant pigment in phosphatidylcholine vesicles was dried under nitrogen gas overnight on an internal reflection element (IRE) made of ZnSe. The adsorbed material was then perfused in the dark with a solution of freshly prepared G_t (8−12 μM). After equilibration of the membrane stacks with G_t (at 17 °C), the sample was illuminated with a 150 W light source through fiber optics for 30 s. The IR absorption of hydrated phosphatidylcholine was determined by drying protein-free vesicles on the IRE and then adding water. A pure water spectrum measured in the same cell was used as reference. All experiments were carried out with a Bruker IFS 28 apparatus equipped with a nitrogen-cooled MCT detector. Interferograms (256 scans co-added) were recorded with scan velocity 11 at 2 cm^{-1} resolution. The intensity reaching the detector was reduced by a 2000 cm^{-1} cutoff Ge filter. A nonlinearity correction was applied to all interferograms using Bruker software.

RESULTS

Figure 1A and 1B show the signal associated with G_t binding in the dark to adsorbed vesicles containing reconstituted rhodopsin or mutant pigment E134Q, respectively. Complete loading of G_t into the stacks of lipid vesicles is essential for the identification of G_t-dependent absorption changes in light-induced difference spectra of MII−G_t complexes. Efficient loading of the vesicles with G_t in the dark minimizes additional G_t uptake during the spectral acquisition time after illumination. Spectra recorded 1 h after G_t addition are ratioed against those measured 3 min after G_t addition. The absorption increase in the amide I and II range that indicates binding of G_t to vesicles in the dark was essentially complete after 20 min. Using the absorption 3 min after addition of G_t as reference eliminated baseline perturbations that accompanied injection of buffer containing G_t into the sample compartment. Prominent absorption increases are noted in the amide I and II spectral regions at 1640 and 1550 cm^{-1}, respectively. In addition, an absorption increase is seen at 1401 cm^{-1} that is most likely attributable to the symmetric COO^- stretching vibrations of carboxylic acid residues of G_t, which are expected to be unprotonated at pH 6.8. These spectral features correspond very well to those of G_t binding to disk membranes in the dark using the identical experimental setup (*16*). Therefore, association of G_t with lipid vesicles containing rhodopsin mutant E134Q is not perturbed by the single amino acid substitution despite evidence that Glu^{134} might be involved in the stabilization of rhodopsin structure in reconstituted vesicles (*13*). The absorption decreases in the frequency range of carbonyl stretching (1700−1800 cm^{-1}) and lipid headgroup modes (900−1300 cm^{-1}) are attributed to swelling of the adsorbed membranes associated with G_t binding (*16*).

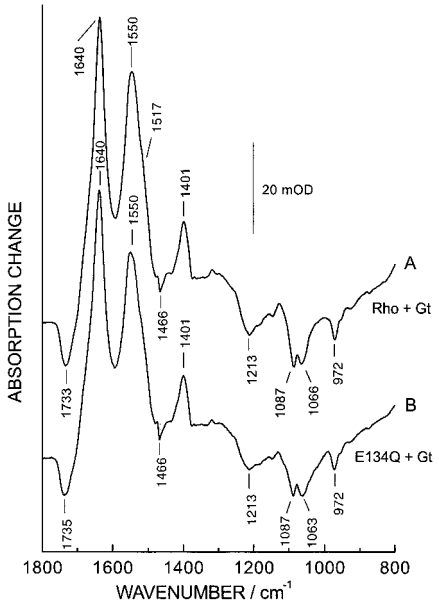

FIGURE 1: Infrared spectral changes during binding of G_t to phosphatidylcholine vesicles reconstituted with Con A-purified rhodopsin (trace A) or mutant rhodopsin E134Q (trace B) in the dark. ATR-FTIR spectra of reconstituted adsorbed vesicles were recorded 3 min and 1 h after the addition of G_t. The difference spectra represent the absorption change between the two times. The spectra were measured at 17 °C [10 mM sodium phosphate buffer, pH 6.8 (A) and 7.1 (B), 200 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT]. Positive bands are due to accumulation of G_t within the vesicle layer at the surface of the IRE, and negative bands are caused by decreasing absorption of phosphatidylcholine.

The spectral changes noted upon G_t binding to E134Q are essentially identical to those obtained upon G_t binding to rod outer segment rhodopsin. The slightly higher lipid carbonyl stretching frequency (1735 cm^{-1} in E134Q versus 1733 cm^{-1} in rhodopsin) may reflect a slightly different degree of incorporation of water into the adsorbed layer of vesicles. Variations in vesicle size and packing during the drying process may affect the swelling behavior of the adsorbed sample when buffer is added. The detailed assignment of bands arising from the adsorption of phospholipid vesicles to the IRE is not directly germane to the present results and will be described in detail elsewhere (Fahmy, K., in preparation).

The ratio of the amide I (1640 cm^{-1}) to amide II (1550 cm^{-1}) absorption bands is slightly higher for the E134Q pigment compared with that of rhodopsin. This difference is most likely accounted for by experimental variations in the orientation of pigment and G_t with respect to the normal of the IRE surface. Partial replacement of water by G_t may also affect the net absorption increase of the amide I band

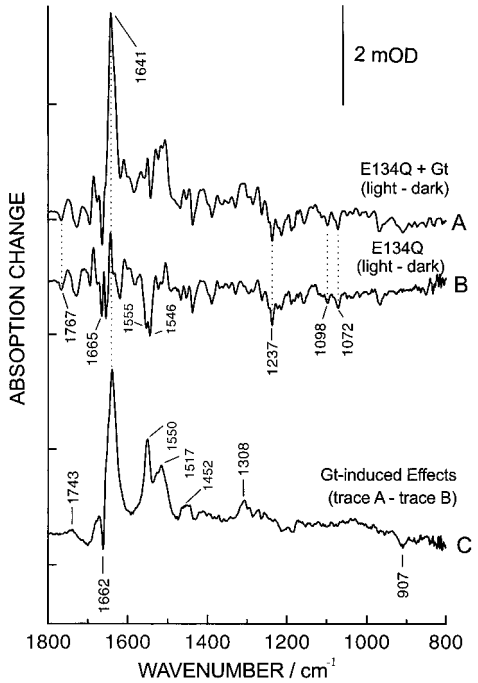

FIGURE 2: ATR-FTIR difference spectra of mutant pigment E134Q reconstituted into phospholipid vesicles. Light-induced changes are recorded. Trace A, ATR-FTIR difference spectrum of mutant pigment E134Q in the presence of G_t. Trace B, ATR-FTIR difference spectrum of mutant pigment E134Q in the absence of G_t. Trace C, Absorption changes induced by complex formation between G_t and mutant pigment E134Q calculated by spectral subtraction (trace A − trace B). The spectra were measured at 17 °C (10 mM sodium phosphate buffer, pH 7.1, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT).

because an absorption decrease of the strong water absorption band at 1640 cm^{-1} (H−O−H scissoring) may superimpose with the amide I band. The magnitude of this effect would again depend on microscopic packing properties of the vesicles. Taking into account these potential physical variables, the time course, magnitude, and frequencies of absorption changes that accompany binding of G_t to the vesicles reconstituted with E134Q in the dark agree extremely well with results obtained with wild-type rhodopsin in phosphatidylcholine vesicles and in disk membranes (*16*).

The effects of illumination of mutant E134Q are presented in Figure 2. Figure 2, trace A shows the ATR-FTIR difference spectrum of membranes containing E134Q that were preincubated with G_t, and trace B shows the spectrum of membranes containing E134Q in the absence of G_t. The magnitude of the light-induced absorption changes is small compared with the changes caused by G_t binding in the dark as indicated by the OD scale bar. These data show that spectral markers of G_t dark binding to membranes and pigment photoactivation can be probed separately in the ATR cell.

Mutant E134Q exhibits reduced sensitivity to alkaline pH with respect to MII formation. Consequently, in the difference spectrum of mutant E134Q in Figure 2B, the contribution from MII should be even more than the 65% reported to form from wild-type rhodopsin under similar conditions (*23*). In addition, residual absorption of the chromophore at 950 cm^{-1} in the MI-like spectrum of E134Q is even smaller than that reported in the difference spectra of reconstituted wild-type rhodopsin recorded at the more acidic pH of 6.8 where MII formation was ∼70% MII (*16*, *24*, *25*). The E134Q replacement does not distinctly influence bands in the 1700−1800 cm^{-1} range, which includes the C═O stretching modes of protonated carboxylic acid groups and lipid esters. This result agrees with the lack of distinct effects on carbonyl stretching modes during MII formation in Glu^{134} replacement mutants expressed in *Sf*9 cells (*13*).

The light-induced difference spectrum of E134Q shows essentially all of the bands characteristic of wild-type rhodopsin (*16*). Only small alterations are noted in the amide I spectral range. For example, the 1662 cm^{-1} negative band of dark rhodopsin is shifted to 1665 cm^{-1} in E134Q, and the neighboring negative band at 1654 cm^{-1} is reduced by about 50%. The introduction of one additional amide group in the side chain of Gln^{134} in the E134Q mutant might contribute to the small alterations in the amide I spectral range. However, the magnitude of the effect on the band at 1654 cm^{-1} appears too large to be caused by a single amide group. Since the E134Q pigment activates G_t more efficiently than wild-type rhodopsin, the spectral alterations cannot be related to a functional defect in G_t activation. In addition, the prominent MII absorption at 1641 cm^{-1} is present, which is generally displayed by active photoproducts but is absent in an inactive form of another recombinant pigment (*27*). The deviations from the wild-type MII difference spectrum, especially the reduced 1654 cm^{-1} band, suggest a "partially active" receptor conformation (*28*, *29*) that exists already in the dark state of E134Q (*30*), thereby reducing additional conformational changes apparent during photoactivation.

The effects of G_t on light-induced absorption changes in the E134Q pigment are shown in Figure 2, trace A. The predominant effects are the increased absorption at 1641 cm^{-1} and the superposition of the negative amide II and C═C stretching bands of the dark state with a broad absorption increase between 1500 and 1560 cm^{-1}. These differences, as well as more subtle spectral changes, are resolved when the difference spectrum of E134Q recorded in the absence of G_t is subtracted from that obtained in the presence of G_t (Figure 2, trace C). The subtraction constant was chosen so that the negative band of Asp^{83} at 1767 cm^{-1} cancels in the resulting spectrum. A thorough analysis of G_t-induced bands showed that extra MII caused positive bands at 1237 and 968 cm^{-1} in a pH-dependent manner and that these bands vanished when the MII:MI ratio of the reference spectrum matched that formed in the presence of G_t. The influence on the 1735 cm^{-1} absorption increase, however, was negligible when 65−70% MII was present in the reference spectrum (*16*). At pH 7.1, mutant E134Q forms more than 70% MII even in the absence of G_t, thereby reducing its ability to form extra MII. Correspondingly, bands at 1237 and 968 cm^{-1} are barely resolved in the G_t-induced absorption changes of mutant E134Q when the reference spectrum is recorded at the same pH. Therefore, the resulting spectral features in trace C are largely dominated by G_t-induced absorption changes. Distinct G_t-induced bands are seen at 1640, 1550, and 1517 cm^{-1}. Bands at identical positions are observed with disk membranes and with wild-type reconstituted rhodopsin. Also, the less pronounced absorption changes at 1308 and 907 cm^{-1} are in excellent agreement with those previously described. Therefore, the data confirm that the amino acid replacement does not interfere with the productive interaction of MII with G_t.

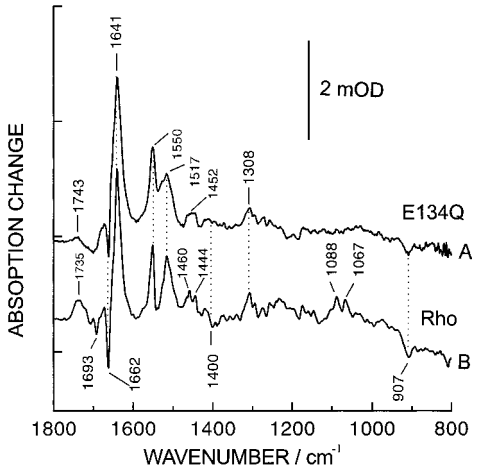

FIGURE 3: Comparison of the effect of G_t binding on light-induced spectral changes in reconstituted phosphatidylcholine vesicles containing recombinant pigment E134Q (trace A) or rhodopsin (trace B). Absorption changes induced by complex formation between G_t and pigment were calculated by spectral subtraction as in Figure 2. The spectra were measured at 17 °C [10 mM sodium phosphate buffer, pH 7.1 (A) and 6.8 (B), 200 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT]. The reference spectrum used for generation of trace B was recorded at pH 5.5 to compensate for extra MII formation by reconstituted purified rhodopsin.

The difference spectra of E134Q and rhodopsin in the presence of G_t are directly compared in Figure 3. Spectral changes are noted at frequencies typical of vibrations of carboxylic acid groups. These alterations can be directly related to the removal of the carboxylic acid group in the side chain of Glu^{134} in the mutant pigment. Based on previous experiments carried out in D_2O, the low-frequency half of the broad absorption increase at 1735 cm^{-1} (Figure 3, trace B) was assigned to protonation of an external carboxylic acid group in the wild-type MII−G_t complex. Correspondingly, the negative band at 1400 cm^{-1} was attributed to the symmetric COO^- stretching mode of this group in the ionized state in dark rhodopsin. Upon E134Q mutation, both of these spectral features vanish (Figure 3, trace A). This result allows the assignment of the 1400 cm^{-1} (negative) band to ionized Glu^{134} in dark rhodopsin, and the 1735−1725 cm^{-1} (positive) band to protonated Glu^{134} in the light-activated MII−G_t complex. The complete absence of the 1400 cm^{-1} band argues for Glu^{134} being the sole carboxylic acid group that becomes protonated in MII−G_t.

A lipid ester carbonyl stretching mode was suggested to cause the high-frequency part of the broad 1735 cm^{-1} band in rhodopsin (Figure 3, trace B) because of its persistence in D_2O-containing samples (*16*). The composite nature of the 1735 cm^{-1} absorption is confirmed here, since removal of Glu^{134} abolishes only part of this band, as is obvious from the residual absorption increase at 1743 cm^{-1} (Figure 3, trace A). The frequency of this band is 2 cm^{-1} higher than that of the corresponding band of wild-type MII−G_t in D_2O, and its magnitude is reduced, indicating that the perturbation of a lipid ester C═O stretching mode is altered by the amino acid replacement. An involvement of absorption changes from lipids interacting with MII−G_t is not unlikely. A specific interaction of rhodopsin with a lipid in disk membranes has recently been described and causes a difference band at 1743 cm^{-1} (positive)/1724 cm^{-1} (negative) (*31*). Therefore, it is of interest to analyze other spectral regions in which lipids may contribute to absorption changes during MII−G_t complex formation. In disk membranes as well as in reconstituted vesicles, G_t induces a structured absorption increase in the spectral range between 1440 and 1470 cm^{-1} with peaks at 1460 and 1444 cm^{-1} (Figure 3, trace B). In mutant E134Q, however, G_t induces an unstructured absorption increase at 1450 cm^{-1}.

The presence of G_t also causes the appearance of absorption bands in the range of the symmetrical PO^{2-} and C−O−PO^{2-} vibrations between 1050 and 1100 cm^{-1}. In disk membranes (*16*) and reconstituted vesicles, MII−G_t formation is accompanied by small but reproducible absorption increases at 1088 and 1067 cm^{-1} (Figure 3, trace B). Dark rhodopsin (*16*) and the dark state of E134Q exhibit absorption bands in the same frequency range (Figure 2). However, the peaks at 1098 and 1072 cm^{-1} differ significantly from those induced by G_t. Therefore, it can be excluded that the bands at 1088 and 1067 cm^{-1} are artifacts caused by spectral subtraction. These bands are abolished in the difference spectrum of MII−G_t of E134Q. In the native complex, they may be caused by the phosphate ester of the GDP bound to G_t. However, the frequencies of these bands match those of the phosphate ester stretching modes of phosphatidylcholine in fully hydrated reconstituted vesicles. Therefore, a tentative assignment of the 1067 and 1088 cm^{-1} bands to lipid headgroups that become perturbed during formation of MII−G_t seems reasonable. Thus, the entire ensemble of G_t-induced bands at 1743, around 1450, and between 1060 and 1100 cm^{-1} may indicate that lipid absorptions are influenced by the E134Q replacement.

DISCUSSION

Light-induced formation of the complex between G_t and the recombinant pigment E134Q was probed by ATR-FTIR difference spectroscopy and compared with the corresponding spectrum of wild-type rhodopsin. Two distinct spectral alterations were observed in the E134Q mutant: (i) the lack of a C═O stretching vibration around 1730 cm^{-1} (positive), and (ii) the lack of a band at 1400 cm^{-1} (negative) typical of a symmetric COO^- stretching mode. These spectral bands can be assigned to Glu^{134} in rhodopsin. The data show that the equilibrium of the protonation reaction of Glu^{134} is shifted from an ionized state in dark rhodopsin (1400 cm^{-1} absorption) to a protonated state in MII−G_t (1730 cm^{-1} absorption). Light-induced protonation of this residue has been suggested based on the inhibition of proton uptake by MII (*9*) and the pH sensitivity of G_t activation of the detergent-solubilized E134Q pigment (*5*). In rhodopsin, only the protonated form of MII, MII_b, interacts with G_t (*32*). Therefore, stabilization of MII by G_t, "extra-MII formation" (*33*), specifically populates the MII_b state and depopulates the unprotonated MII_a species. This explains why specific infrared absorption changes of MII_b may become visible only in the presence of G_t. Our data show that protonation of Glu^{134} is favored in the MII−G_t complex and may thus be linked to MII_b, rather than MII_a formation. These results strongly suggest that Glu^{134} functions as a proton acceptor in the MII_a to MII_b transition in the absence of G_t. Accordingly, the lack of distinct spectral alterations between 1700 and 1800 cm^{-1} and near 1400 cm^{-1} in the difference spectrum of mutant E134Q alone (*13*) argue for predominant formation of MII_a under

the conditions of previous FTIR experiments in the absence of G_t.

In a recent polarized ATR-FTIR study on MII formation, however, bands at 1736 and 1403 cm^{-1} have been tentatively assigned to the C═O stretching and COO− stretching modes of Glu^{134}, respectively (*15*). Our results provide direct evidence for the protonation change at this site in the presence of G_t. The data do not support a previous assignment of a band at 1704 cm^{-1} to the C═O stretching mode of Glu^{134} in MII−G_t (*14*). In addition, we show here that the Glu^{134} replacement gives rise to spectral alterations that may be caused by lipid headgroups and side chains in MII−G_t. Lipid−protein interactions in MII are apparently modulated by G_t and probably involve the environment of Glu^{134}, extending the observation that a lipid carbonyl band shifts from 1724 to 1743 cm^{-1} during MII formation in vesicles (*31*).

Conclusions from our data with respect to a putative proton uptake function of Glu^{134} depend on the validity of present models describing different protonation states of MII_a and MII_b as well as their affinities for G_t. In a recent study, proton uptake by MII_a rather than MII_b has been suggested to proceed concomitantly with Schiff base deprotonation (*34*). The conflicting models render an unequivocal assignment of the protonation of Glu^{134} to the different MII species in the absence of G_t impossible. Irrespective of the direct or indirect role of Glu^{134} in proton uptake by MII, the protonation of Glu^{134} demonstrated here in MII−G_t shows that the neutral side chain at this position favors the active receptor conformation. This agrees with the higher rate of G_t activation by the mutant pigment E134Q and the constitutive activity of several mutant opsins with Glu^{134} replacements (*6, 7*). Anticipation of the neutral state of the Glu^{134} side chain in the mutant may thus induce an active-like conformation in a restricted domain on the rhodopsin surface in the dark, assuming that separate activating transitions occur in receptor subdomains (*28, 29*). The reduction of a negative band at 1654 cm^{-1} in the MII difference spectrum of E134Q may indicate a reduction of additional conformational changes in such a "pre-activated" domain. Reduction of difference bands has been related to partial preactivation in another pigment (*35*) and in a constitutively active opsin mutant (*36*).

Protonation of Asp^{142} in the α_{1b} adrenergic receptor, which is homologous to Glu^{134} in rhodopsin, also promotes the formation of an active state (*37*). It has been suggested that protonation of Asp^{142} in the α_{1b} adrenergic receptor may be linked to exposure of the carboxylic acid side chain to the lipid phase (*38*). In light of this model, the correlation of MII−G_t absorptions and lipid headgroup vibrations revealed by the E134Q mutant is striking. Among the mutation-sensitive bands that coincide with absorption frequencies of phosphatidylcholine, the 1067 and 1088 cm^{-1} vibrations might arise from phosphate stretching modes of GDP. However, absorption from GDP would be expected to decrease rather than to increase because GDP is released from G_t in the complex with MII. Consequently, GDP should contribute less to the attenuation of the IR beam in the evanescent field. Tentative assignment of these bands to phosphate stretches of fully hydrated phosphatidylcholine is more reasonable, and the data suggest that a specific interaction of Glu^{134} with lipid headgroups may be of functional importance during G_t binding and activation. Topologically, such an interaction appears possible because Glu^{134} is positioned at the interface between the lipid layer and the cytoplasm (*39*). Rigid body movements of TM helix 3 relative to TM helix 6 (*40*) and conformational changes in the Glu-Arg-Try motif itself may cause a transfer of the Glu^{134} side chain from a polar to a nonpolar lipid environment, thereby inducing its protonation. Alternatively, charge neutralization at this site by substitution of Gln for Glu would anticipate a lipid helix packing in which the Gln side chain points to an apolar environment already in the dark state. A further perturbation of lipid vibrations during MII−G_t formation would be reduced. This may explain the lack of spectral changes between 1060 and 1090 cm^{-1} and the reduced absorption increase at 1743 cm^{-1} upon MII−G_t formation in E134Q.

In summary, the results demonstrate that protonation of Glu^{134} occurs in the MII−G_t complex, giving rise to a C═O stretching mode at 1730 cm^{-1} (positive) and a symmetrical carboxylate stretch at 1400 cm^{-1} (negative). This protonation may be linked to specific lipid−protein interactions in the MII−G_t complex. Additional studies are underway to characterize specific lipid−protein interactions during receptor-dependent G protein activation. The high degree of conservation of an acidic amino acid in GPCRs at a position homologous to Glu^{134} of rhodopsin (*41*) hints at a common mechanism for G protein activation that may operate by protonation of the Asp(Glu) side chain in the conserved Asp(Glu)ArgTyr(Phe) motif. Although effects of homologous amino acid replacements in other GPCRs are not uniform (*42*−*44*), our data strongly support protonation-dependent signaling in other rhodopsin-like GPCRs as proposed for α_{1b}- and β_2-adrenergic receptors (*2, 38*).

ACKNOWLEDGMENT

We thank J. Isele for helpful discussions and B. Mayer for assistance with G_t purifications.

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BI000912D