A Conserved Carboxylic Acid Group Mediates Light-dependent Proton Uptake and Signaling by Rhodopsin

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A carboxylic acid residue is conserved at the cytoplasmic border of the third transmembrane segment among nearly all G protein-coupled receptors. In the visual receptor rhodopsin, replacement of the conserved Glu134 by a neutral glutamine results in enhanced transducin activation. Here we show that a key event in forming the active state of rhodopsin is proton uptake by Glu134 in the metarhodopsin II (MII) photoproduct. Site-directed mutants E134D and E134Q were studied by flash photolysis, where formation rates of their photoproducts and rates of pH change could be monitored simultaneously. Both mutants showed normal MI1 formation rates. However, E134D displayed a slowed rate of proton uptake and E134Q displayed a loss of light-induced uptake of two protons from the aqueous phase. Thus, Glu134 mediates light-dependent proton uptake by MII. We propose that receptor activation requires a light-induced conformational change that allows protonation of Glu134 and subsequent protonation of a second group. The strong conservation of Glu134 in G protein-coupled receptors implies a general requirement for a proton acceptor group at this position to allow light- or ligand-dependent receptor activation.

Rhodopsin, the photoreceptor of the vertebrate retina rod cell, is a member of the G protein-coupled receptor superfamily. The chromophore in rhodopsin (\( \lambda_{max} = 500 \text{ nm} \)), 11-cis-retinal, is covalently bound via a protonated Schiff base. Light causes photoisomerization of the chromophore and receptor activation. The receptor in its active conformation, \( R^* \), catalyzes guanine nucleotide exchange in the G protein, transducin. Receptor activation requires deprotonation of the retinal Schiff base linkage to form metarhodopsin II (MII) (1–3). MII contains an all-trans-retinal chromophore with an unprotonated Schiff base linkage. Schiff base deprotonation can be monitored spectrally by measuring the increase in absorbance at 380 nm, which corresponds to the formation of MII (\( \lambda_{max} = 380 \text{ nm} \)). In addition, light-induced proton uptake by rhodopsin (4, 5) occurs in a spectrally silent transition after Schiff base deprotonation (6). Thus, the MII state was further characterized as MII1 and MII2 to indicate the states before and after proton uptake, respectively (6). It was suggested that this proton uptake is required for the \( R^* \) conformation (6). It was also reported that a highly conserved carboxylic acid group on the cytoplasmic surface of rhodopsin (Glu134) regulates \( R^* \)-transducin interaction, and that Glu134 in the protonated form favors the binding of \( R^* \) to transducin (7).

In the present report, rhodopsin and site-directed rhodopsin mutants were studied by a flash photolysis method designed to allow simultaneous measurement of the formation of the 380-nm photoproduct and proton uptake or release. We show that the conserved residue Glu134 is directly involved in light-dependent protonation of rhodopsin and argue that this molecular event is required for receptor activation. The strong conservation of Glu134 in G protein-coupled receptors implies a general requirement for a proton acceptor group at this position to allow light- or ligand-dependent receptor activation.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mutant Pigments—**Mutants were constructed in a synthetic gene (8) as described previously (9). Recombinant opsin and mutants were expressed in COS cells (10), regenerated with 11-cis-retinal, and purified by an immunoaffinity procedure in dodecyl malto-side detergent solution (11). The peptide used to elute the pigments from the immunoaffinity resin was removed as described previously (12). The absorption coefficients of the mutant pigments at their absorption maximum (\( \lambda_{max} \)) values (500 nm) were identical to that of rhodopsin (42,700 cm\(^{-1} \text{M}^{-1} \)) (9).

**Flash Photolysis Measurements of Proton Uptake or Release—**Flash photolysis measurements were carried out in a two-wavelength flash photometer previously used by Emeis et al. (1) and by Franke et al. (13). The absorption change of pH indicators bromcresole purple (595 nm) for pH 6 and pH 7 or cresole red (575 nm) for pH 8, respectively, due to proton uptake or release were simultaneously recorded in a two channel digital oscilloscope (Nicolet-2090). To correct the proton uptake signal (\( \Delta H^+ \)) for photoproduct formation at 595 or 575 nm (\( \sim 10\% \)), two aliquots were measured for each sample, one unbuffered and one buffered control. The buffered samples for the control measurement contained in addition 50 mM MES (pH 6), 50 mM HEPES (pH 7), or 60 mM Bis-Tris (pH 8). The difference from the control is the pure \( \Delta H^+ \) value. The contribution of \( \Delta H^+ \) to the MII signal in the unbuffered sample is negligible because bromcresole purple and cresole red show no significant absorption change at 380 nm. The \( \Delta H^+ \) values were calibrated by adding small amounts of HCl (4.3 \( \mu \text{M} \)) to the dark sample. The resulting absorption change was then compared to the flash-induced change measured on the same sample. Samples were photolyzed by flashes of green light (490–500 \( \pm 5 \text{ nm} \) band-pass filter, 20-\( \mu \text{s} \) flash) where the mole fraction of bleach/flash was 20%. All samples contained 0.9 mM rhodopsin (native or mutant), 0.4 mM dodecyl malto-side detergent, 130 mM NaCl, and 12.3 mM bromcresole purple (for measurements at pH 6 and 7) or 18 \( \mu \text{M} \) cresole red (for measurement at pH 8). Figs. 1 and 2 show the results of individual experimental traces. Each experiment was reproduced with samples from independent preparations.

**RESULTS AND DISCUSSION**

Mutant pigments E134D and E134Q, in which Glu134 was replaced by Asp and Glu, respectively, were prepared in parallel with bovine rhodopsin using an established COS-cell expression procedure (7, 8, 10). A two-wavelength flash photometer
Rhodopsin (6). Mutant E134D showed light-dependent protonation changes (H+/MII) as a function of pH (Fig. 3). At unaffected final amplitude for E134D.

The kinetics of MI1 formation were slower kinetics. In contrast, mutant E134Q displayed a lack of uptake of less amplitude at pH 7, and a small proton release at pH 8. This result essentially reproduced the findings obtained in Fig. 2 with greater time resolution, demonstrating, in particular, the reduction of the proton signal rise time and the unaffected final amplitude for E134D.

The amplitudes of the pH-sensitive absorption changes are normalized and plotted to show the magnitude of the respective mutant pigments. These changes similar to those of native rhodopsin, but with the conditions of the assay, the kinetics of MI1 formation were independent of pH in the range of 6–8 for rhodopsin. The mutants pigments exhibited the same kinetics of MI1 formation as did rhodopsin (data not shown). The change in the protonation state of the pigments was monitored simultaneously with MI1 formation by following the absorption change of a pH-sensitive dye as described (6) (Fig. 1, lower panel). Rhodopsin showed a rapid uptake of protons in response to light at pH 6, a slower uptake of less amplitude at pH 7, and a small proton release at pH 8. This result essentially reproduced the findings obtained with dodecyl maltoside-solubilized bovine rod outer segment rhodopsin (6). Mutant E134D showed light-dependent protonation changes similar to those of native rhodopsin, but with slower kinetics. In contrast, mutant E134Q displayed a lack of proton uptake at pH 6, a small net release of protons at pH 7, and a normal proton release at pH 8. The kinetics of proton uptake in rhodopsin, E134Q, and E134D at pH 6 are compared in Fig. 2 with greater time resolution, demonstrating, in particular, the reduction of the proton signal rise time and the unaffected final amplitude for E134D.

The amplitudes of the pH-sensitive absorption changes are normalized and plotted to show the magnitude of the respective protonation changes (H+/MII) as a function of pH (Fig. 3). At acidic pH, two protons are taken up by native rhodopsin and mutant E134D. Surprisingly, the removal of the single carboxylic acid side chain of Glu(34) in mutant E134Q abolished the uptake of both protons. The proton release common to all three pigments, which has already occurred in mutant E134Q by pH 7, as compared to pH 8 in the two other pigments, is most likely a consequence of Schiff base deprotonation upon MI1 formation. The complete blockade of proton uptake in E134Q is expected to abolish the pH dependence (14, 15) of the spectrally defined MI1 form (the sum of 380-nm MI1, and MI2, forms in the coupled equilibria, MI → MI1 ↔ MI2, where MI is metarhodopsin I) as has been observed in digitonin solutions of this mutant (16).

The results show that the carboxylic acid side chain of Glu(34) in the tripeptide sequence (Glu/Arg/Tyr) near the cytoplasmic border of helix C, which is conserved among most G protein-coupled receptors (17–19), is likely to mediate the light-dependent proton uptake by rhodopsin. It is suggested that the side chains of Glu(34) and an additional unidentified group become protonated at neutral pH during the MI1 to MI2 transition. Protonation of both sites does not seem to be coupled to the Schiff base deprotonation, which was not affected by the mutations. This supports an earlier suggestion that proton uptake occurs in a hydrophilic domain separate from the hydrophobic retinal binding site (6). Here, Glu(34) has been identified as part of this surface domain. The results obtained from the two mutants suggest in particular that Glu(34) is the primary protonation site for proton uptake after Schiff base deprotonation, but before MI1 formation. The abolishment of the uptake of two protons in E134Q can be explained if protonation of Glu(34) is required to facilitate the second proton transfer to rhodopsin.

In thermodynamic terms, the protonation of Glu(34) would lead to a transient state in which the activation energy for protonation of the second site in the MI1 to MI2 transition is low enough to proceed with the observed rate. This notion is strongly supported by the E134D mutation, which affects the rate of proton uptake but not the two proton stoichiometry. This is in excellent agreement with the observation that in rod outer segment rhodopsin a single protonation (here assigned to Glu(34)) accelerates the total uptake of two protons in the MI1 to MI2 transition (6). Such a mechanism also agrees with the hypothesis that the protonation state of Glu(34) affects other titratable surface residues on rhodopsin and that its neutralization allows efficient signaling (7). For example, mutant E134Q showed enhanced light-dependent activation of transducin at alkaline pH (7). The same mutant also showed constitutive activity in the absence of chromophore (20). Because of the shape of the pH rate profile of the E134Q apoprotein, it was suggested that the mutation had affected mainly one of two
measurable pKₐ values for constitutive activation (20). The present results indicate that light-dependent R* formation may depend on a more complex mechanism, which involves the protonation of at least two different amino acid side chains.

We propose here that protonation of Glu¹³⁴ is necessary to induce a completely active receptor conformation. However, proton uptake is not sufficient for receptor activation, since the mutant E134D shows a normal magnitude of proton uptake but activates transducin less efficiently than rhodopsin (7, 9).

Given the direct involvement of the conserved tripeptide and the adjacent cytoplasmic loop in light-dependent transducin activation (7, 9, 13, 21), the Glu¹³⁴-mediated regulation of surface charges by proton uptake may be relevant to a general mechanism of G protein activation and may provide a direct functional correlate of the structural change in this region probed by electron paramagnetic resonance of rhodopsin spin-labeled at Glu¹³⁴ (22, 23). Elucidation of the light-dependent trigger mechanism that may link chromophore isomerization to R* formation by intramolecular proton movement is the goal of future work.

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