Regulation of the Rhodopsin–Transducin Interaction by a Highly Conserved Carboxylic Acid Group†

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Received February 1, 1993; Revised Manuscript Received April 20, 1993

ABSTRACT: Rhodopsin is a member of a family of G protein-coupled receptors which share structural and functional homologies. A tripeptide sequence (Glu or Asp/Arg/Tyr) at the cytoplasmic border of the third transmembrane segment is conserved among most of these receptors. This region is involved in G protein activation in rhodopsin as well as in other receptors. The role of the conserved Glu-134 was studied by site-specific mutagenesis of rhodopsin in combination with a real-time fluorescence assay of G protein (transducin) activation. Assay conditions were chosen under which the transducin activation rate was determined either by rhodopsin–transducin complex formation or by GTPγS-induced complex dissociation. Glu-134 was replaced by Gln in order to mimic the protonated state of the carboxylic acid group. This mutation caused the pH dependency of complex formation to extend to the alkaline range as compared with rhodopsin. Replacement of Glu-134 by Asp had an opposite but less pronounced effect on the pH dependency and lowered the overall efficiency of transducin activation. The acidity constant (pK_a) of the residue at position 134 did not directly determine the pH sensitivity of complex formation, indicating that other amino acid residues contribute to a titratable binding domain that includes Glu-134. In contrast, the pH sensitivity of GTPγS-induced complex dissociation was not changed by the mutations, although absolute rates were affected. The data suggest that the protonated state of Glu-134 favors binding of rhodopsin to transducin and that Glu-134 is not titratable in the rhodopsin–transducin complex.

Rhodopsin is the visual photoreceptor of the rod cell. It is a member of the superfamily of seven transmembrane helix receptors that activate guanine nucleotide-binding regulatory proteins (G proteins).[1] The 11-*cis*-retinal chromophore is covalently linked via a protonated Schiff base at Lys-296. Photoisomerization of the chromophore to the all-trans form results in the formation of metarhodopsin II (MII) that catalyzes the activation of guanine nucleotide exchange in the rod cell G protein transducin. The photoactivated form of rhodopsin is also widely referred to as R*. Formation of R* requires Schiff base deprotonation (Longstaff et al., 1986).

The transmembrane domains of rhodopsin have been postulated to have predominantly α-helical secondary structure (Dratz & Hargrave, 1983). In particular, the helical structure of the third transmembrane segment has been supported by several lines of evidence, including the assignment by site-directed mutagenesis of helix borders (Sakmar et al., 1989; Zhukovsky & Oprian, 1989) and the movement of the retinylidene Schiff base counterion by one helix turn (Zhukovsky et al., 1992; Zvyaga et al., 1993). A tripeptide sequence (Glu or Asp/Arg/Tyr) at the cytoplasmic border of the third transmembrane segment is conserved among most G protein-coupled receptors (Attwood et al., 1991; Dohlman et al., 1991). This region is involved in G protein activation in rhodopsin (Franke et al., 1989, 1992; Sakmar et al., 1989) as well as in other receptors (Fraser et al., 1988). Mutations of the conserved arginine in rhodopsin (Arg-135) abolish transducin activation (Franke et al., 1989, 1992; Sakmar et al., 1989). However, mutations of the conserved glutamic acid residue (Glu-134) have variable effects on transducin activation. Replacement of Glu-134 by glutamine (mutant E134Q)[2] resulted in a hyperactive mutant, and replacement by Asp (E134D) resulted in a mutant which activated transducin less efficiently (Sakmar et al., 1989). These mutations remove or exchange a protonatable amino acid, respectively. Therefore, the effect on transducin activation may be caused by alterations of the net surface charge at the cytoplasmic face of rhodopsin near residue 134. If so, one would expect that pH-induced changes of the protonation state of the titratable group(s) are affected by the amino acid replacements. To test this hypothesis, we have measured the pH dependency of transducin activation rates for the mutants E134Q and E134D by employing a spectrofluorometric assay which monitors nucleotide exchange in transducin. Assay conditions were chosen to study the effects of pH on rhodopsin–transducin complex formation as well as on nucleoside triphosphate-induced complex dissociation. The data show the role of Glu-134 in regulating the pH dependency of transducin binding. Glu-134 also influences the nucleotide exchange rate but does not determine the pH sensitivity of this particular reaction step in the signalling pathway.

MATERIALS AND METHODS

Materials. Sources of most materials have been previously reported (Franke et al., 1992; Sakmar et al., 1989, 1991). Nucleotides were purchased from Boehringer Mannheim.

Preparation of Rhodopsin Mutants and Transducin. Mutants were constructed in a synthetic gene (Ferretti et al.,

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[1] Abbreviations: G protein, guanine nucleotide-binding regulatory protein; MI, metarhodopsin I; MII, metarhodopsin II; R*, photoactivated rhodopsin.

[2] Mutants are designated by the native amino acid residue (single-letter code) and its position number followed by the substituted amino acid residue. For example, in mutant E134Q, Glu-134 is replaced by Gln.

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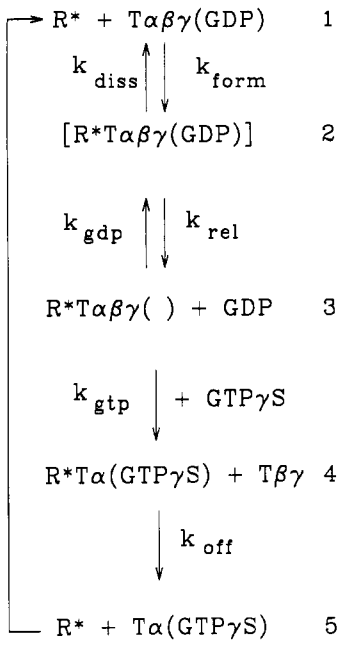

FIGURE 1: Reaction scheme for light-induced transducin activation by rhodopsin. The fluorescence assay employed in this study is based on the observation that the intrinsic fluorescence of G protein α-subunits increases markedly upon binding guanine nucleoside triphosphate. The assay monitors the rate of formation of Tα(GTPγS). As described in the text, the amount of GTPγS added to a mixture of photoactivated rhodopsin and transducin determines the steady-state concentration of the long-lived complex symbolized in line 3. In the presence of 5 μM GTPγS, the rate of Tα(GTPγS) increase was determined by the rate of reactions leading from line 1 to line 3. In the presence of 100 nM GTPγS, the steps from line 3 to line 5 dominated the time course of fluorescence increase.

1986) as previously described (Sakmar et al., 1989). Recombinant opsin and mutants were expressed in COS cells (Oprian et al., 1987), regenerated with 11-*cis*-retinal, and purified by an immunoaffinity procedure in dodecyl maltoside detergent buffer (Franke et al., 1992; Sakmar et al., 1989; Zvyaga et al., 1993). Transducin was purified from bovine retinal rod outer segments by successive washes and hexylagarose chromatography (Fung et al., 1983).

UV–Visible Absorption Spectroscopy. Spectroscopy was performed on a λ-19 Perkin-Elmer spectrophotometer at 10 °C in a cuvette with a 1-cm path length (Chan et al., 1992; Zvyaga et al., 1993). The absorption coefficients of the mutant pigments at their absorption maximum (λ_{max}) values (500 nm) were identical to that of rhodopsin (42 700 cm^{-1} M^{-1}) (Hong & Hubbell, 1972; Sakmar et al., 1989).

Transducin Activation as a Function of GTPγS Concentration. The rhodopsin-catalyzed nucleotide exchange in transducin can be dissected into consecutive steps depicted in Figure 1. The scheme follows essentially the description by Chabre (1985). R* binds to transducin in its GDP-bound form (line 1), resulting in a short-lived transient complex (line 2). Upon release of GDP, a long-lived (hours) rhodopsin–transducin complex is formed (line 3). Binding of GTPγS (line 4) induces dissociation of this complex, thereby releasing activated transducin and R* (line 5). The GTPγS-bound form accumulates and causes the fluorescence increase measured as described below. R* is capable of undergoing the same reactions repeatedly and eventually activates the entire transducin pool. Since the mutants studied in this report are able to activate transducin catalytically, none of the depicted steps is blocked by the amino acid replacement. Therefore, the same scheme describing the influence of GTPγS concentration on the rate at which GTPγS-bound transducin accumulates (line 5) applies both to rhodopsin and to the mutant pigments. It is obvious that at low GTPγS concentrations the rhodopsin-transducin complex in line 3 builds up due to its long intrinsic lifetime. In all experiments, the α-subunit concentration was more than 100-fold higher than that of R*. Therefore, virtually all R* was bound to transducin during the steady-state concentrations reached after the addition of low GTPγS concentrations. In contrast, high concentrations of GTPγS deplete the population of rhodopsin–transducin complexes (lines 2, 3, and 4) due to the fast dissociations in steps 3–5 of the reaction scheme. Therefore, virtually all R* is present in its unbound form during the reaction. Thus, the steady-state concentrations of R* can be shifted between bound and free forms by adjusting the GTPγS concentration. At a given pH, fluorescence time courses were recorded from separate identical samples using high (5 μM) and low (100 nM) concentrations of GTPγS to initiate the reactions. This has allowed the study of the pH dependence of complex fomation ("high" nucleotide, free R*) as compared with the pH sensitivity of GTPγS-induced complex dissociation ("low" nucleotide, bound R*). In addition to GTPγS, the accumulation of rhodopsin–transducin complexes is also affected by GDP concentration (lines 2 and 3). The initial concentration of GDP was identical for all experiments since a single transducin stock solution was used which contained 5 μM GDP. Therefore, results from different experiments could be directly compared. In this study, the effect of mutations on the pH sensitivity of the described reaction steps was expressed on a relative basis by comparison with rhodopsin. Calculation of absolute numbers of any of the involved rate constants was beyond the scope of the present work.

Spectrofluorometric Assay of Transducin Activation. Active transducin α-subunit concentration was precisely determined by measuring the rhodopsin-catalyzed nucleotide-induced fluorescence increase upon addition of different amounts of GTPγS (Figure 2). The fluorescence assay employed was similar to one described previously (Guy et al., 1990; Phillips & Cerione, 1988). Fluorescence was measured with a specially modified SPEX-Fluorolog II spectrofluorometer in the signal/reference mode with excitation at 300 nm (2-nm bandwidth) and emission at 345 nm (12-nm bandwidth). The excitation at 300 nm was chosen in order to circumvent interference with the nucleotide absorbance which was found to alter the effective excitation intensity in the cuvette if fluorescence was excited by 280-nm light. Thereby, distortion of the measured time courses by the change of free nucleotide concentration is prevented. All experiments were carried out at 10 °C using a water circulating bath connected to the cuvette holder in order to slow down the reaction. This has allowed a relatively long signal integration time of 2 s. The reaction mixture (1.6 mL) containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 0.01% dodecyl maltoside, rhodopsin, and 50 μL of transducin stock solution was stirred continuously at maximum speed. Addition of rhodopsin and nucleotide was done by injecting 50 μL of the appropriate solution into the cuvette with a gas-tight syringe kept at 10 °C. After addition of pigment, the sample was continuously illuminated in the cuvette with 543-nm light from a HeNe laser (Melles-Griot) connected to the sample compartment by a fiber optic light guide. Stray light was efficiently blocked from reaching the detector by a double monochromator.

To determine the pH dependence of activation, assays were carried out in 10 mM Tris–maleate buffer adjusted to the

proper pH (Franke et al., 1992). Other buffer components were as given above except that chloride ion concentration varied with pH (referred to as assay buffer). The relative activation rate is defined as the slope of the fluorescence signal after GTPγS addition. These slopes were calculated by linear regressions through the data points covering 40–60 s. Under conditions of the assay, the rate of photobleaching to form a 380-nm species characteristic of metarhdopsin II was identical for the three samples and independent of pH. Photoconversion was complete in 15 s (not shown). R* decay and decay of the photoactivated mutant pigments were negligible. More than half of the initial activation rate was retained after 3 h in the presence of transducin at 10 °C throughout the pH range studied. This finding agrees with previous reports on the stability of the rhodopsin–transducin complex (Bornancin et al., 1989). The effect of pH on relative activation rates was reversible.

RESULTS

Preparation of Mutant Pigments. Two mutant opsin genes were prepared by site-specific mutagenesis. In mutant E134D, Glu-134 was replaced by Asp, and in mutant E134Q, Glu-134 was replaced by Gln. The mutant genes were expressed in COS cells, and the expressed apoproteins were reconstituted with 11-*cis*-retinal chromophore. The resulting pigments were purified in dodecyl maltoside detergent solution. Each of the mutants displayed a λ_{max} value identical to that of rhodopsin prepared in parallel under the same conditions (500 nm). Upon illumination, both mutants formed a species absorbing at 380 nm characteristic of MII. These mutations were chosen because they were reported to have opposite effects on the ability of the resulting pigment to activate guanine nucleotide exchange in transducin (Sakmar et al., 1989). The mutant pigments differ in the number of potentially charged and putatively titratable amino acids on the cytoplasmic border of rhodopsin. A study of the pH dependency of their ability to activate transducin was therefore expected to address the role of the carboxylic acid side chain at position 134 of rhodopsin in the rhodopsin–transducin interaction leading to nucleotide exchange in transducin.

Fluorescence Assay of Transducin Activation by Rhodopsin. A fluorescence assay was developed in order to measure the rate of light-dependent transducin activation by purified recombinant pigments in detergent solution. The assay depends on the observation that the intrinsic fluorescence of G protein α-subunits increases upon guanine nucleoside triphosphate binding, and was similar to those reported previously (Antonny & Chabre, 1992; Guy et al., 1990; Higashijima et al., 1987; Phillips & Cerione, 1988). However, the assay employed for this study was carried out in detergent solution where the concentration of each component (purified recombinant pigment, purified bovine transducin, GDP, and GTPγS) could be determined by spectroscopic methods. As shown in Figure 2, the concentration of active transducin α-subunit could be determined by a fluorometric titration with GTPγS in the presence of rhodopsin. A mixture of transducin and rhodopsin in assay buffer was allowed to equilibrate for 15 min until a stable fluorescence base line was achieved. The photoconversion of rhodopsin to R* did not cause any fluorescence changes. Since the GTP analogue GTPγS is nonhydrolyzable, transducin accumulated in its activated form after successive GTPγS additions indicated by the arrows in Figure 2. Due to the high amount of rhodopsin (15 nM) used in this particular experiment, a new value of constant fluorescence was reached within seconds after each

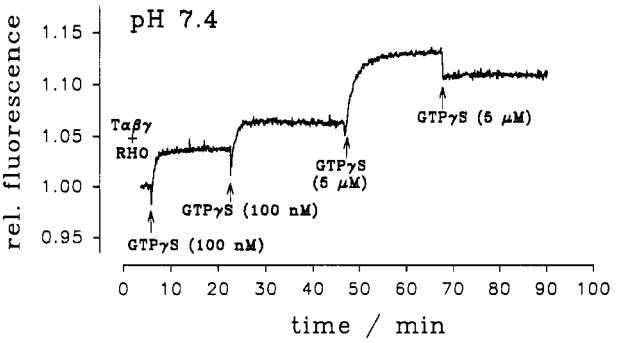

FIGURE 2: Fluorometric titration of purified transducin by nucleotide. A continuous time course of the relative intensity of fluorescence is shown. A mixture of transducin and rhodopsin (15 nM) in 1.5 mL of Tris-HCl buffer (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, and 0.01% dodecyl maltoside was excited at 300 nm, and fluorescence was recorded at 345 nm. Aliquots (50 μL) of GTPγS were added (arrows) to attain the increases in nucleotide concentration as indicated. The lack of fluorescence increase after the last nucleotide addition demonstrates that occupancy of all nucleotide-binding pockets had been achieved during the preceding incremental nucleotide exchanges. From the differences between the plateau values reached after nucleotide addition, a transducin concentration of 360 nM was calculated in this experiment.

GTPγS addition. The UV absorbance of the transducin solution was below 0.2 ODU. Therefore, GTPγS-induced fluorescence increases could be considered proportional to the amount of α-subunits occupied with GTPγS. After correction for sample dilution which caused the initial deflections after GTPγS additions, the fluorescence changes could be correlated with the amount of nucleotide added. After the third GTPγS addition, the entire transducin pool was loaded with nucleotide as shown by the lack of any further fluorescence change upon addition of more GTPγS. Assuming complete nucleotide uptake, an α-subunit concentration of 360 nM was calculated for this experiment. A single transducin preparation thus characterized was employed for an entire set of experiments. For these experiments, the α-subunit concentration was controlled by measuring the UV absorbance of the transducin solution and using the calibration obtained from the fluorometric titration.

Characterization of the pH Dependence of the Rate of Complex Formation between Recombinant Pigments and Transducin. Fluorescence assays of rhodopsin and the mutant pigments were performed in detergent micelles under conditions where the measured rates were proportional to the amount of photoconverted pigment (about 1 nM pigment and 250 nM transducin). Two sets of experiments were carried out which differed in the GTPγS concentration at a given pH. As described under Materials and Methods, the reaction step that determines the rate of transducin activation is the formation of the nucleotide-free rhodopsin–transducin complex (Figure 1, line 3) if GTPγS is added in excess (5 μM) over the α-subunit concentration. Under these conditions at pH 7.4, mutant E134Q was found to activate transducin at a higher rate than rhodopsin, whereas mutant E134D was less active than rhodopsin (Figure 3). The pH dependency curves of activation rates obtained under the same conditions are represented by solid circles in Figure 4. The trace obtained for rhodopsin (Figure 4a) is normalized to the pH optimum observed at about pH 7. All other traces are scaled correspondingly. The rates for transducin activation by rhodopsin decreased by a factor of 2 within approximately 0.5 pH unit to each side of the pH optimum.

A similar pH dependence was obtained for the mutant E134D shown by solid circles in Figure 4b. However, at any

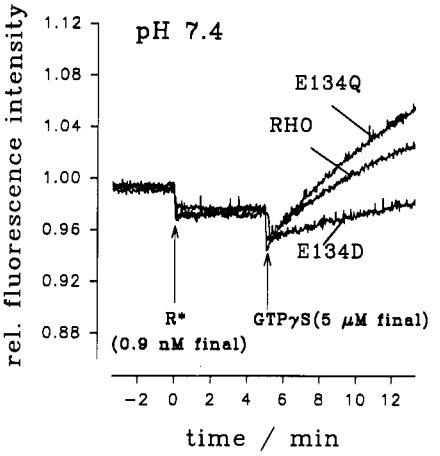

FIGURE 3: Time course of transducin activation by recombinant pigments. The results of separate experiments for rhodopsin, mutant E134D, and mutant E134Q are superimposed. Successive additions of pigment (0.9 nM final) and GTPγS (5 μM final) to a solution of transducin (250 nM) are indicated by arrows. Experiments were carried out under continuous illumination at 10 °C (buffer as in Figure 2). The fluorescence increase after nucleotide addition corresponded to the following activation rates: rhodopsin, 17 pmol/min; mutant E134D, 7 pmol/min; mutant E134Q, 24 pmol/min. The amount of pigment was rate-limiting in each case.

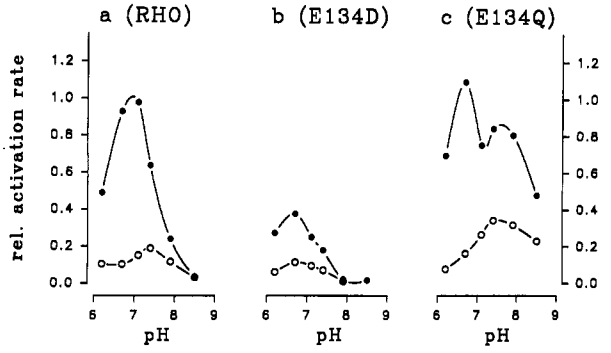

FIGURE 4: Mutational effects on the pH dependence of relative activation rates at different concentrations of GTPγS. The pH dependence of relative activation rates was measured for rhodopsin, mutant E134D, and mutant E134Q at 5 μM (solid circles) and 100 nM (open circles) GTPγS concentrations. Solid circles correspond to conditions where the formation rate of R*–transducin complexes determined the initial rise of Tα(GTPγS) fluorescence. Open circles indicate conditions where complexes could accumulate. The complex dissociation rates determined the transducin activation rate under these conditions (see Materials and Methods). Each point was determined from a fluorescence time course as shown in Figure 3, except that Tris–maleate assay buffer of varying pH was used. The ordinate is scaled so that the maximum activation rate of rhodopsin equals 1.0.

given pH, the reaction proceeded more slowly as compared with rhodopsin-catalyzed transducin activation. The maximal rate obtained with mutant E134D was 40% of the maximal rate for rhodopsin. In addition, a slight shift of the pH optimum to more acidic pH can be inferred for mutant E134D.

Replacement of Glu-134 by Gln caused a much more severe change of the pH sensitivity of rhodopsin–transducin complex formation than did the substitution by Asp. Figure 4c shows that mutant E134Q was able to catalyze nucleotide exchange in transducin at alkaline pH at much higher rates than rhodopsin or mutant E134D. The higher activity reported earlier for mutant E134Q is manifested most distinctly between pH 7.5 and 8.5, a pH range that has previously not been employed in activity determinations based on a GTPase assay. Therefore, the mutant E134Q is best described as a pigment with a catalytic activity that extends further into the alkaline pH range than that of rhodopsin. In addition, a narrow pH optimum is reproducibly observed between pH 6 and 7, slightly exceeding the maximal activity of rhodopsin in this pH range. Only at pH 7.2 was the rate of transducin activation by rhodopsin faster than that for the mutant. The complex shape of the curve in Figure 4c indicates that the rates as a function of pH are not uniformly affected by the amino acid replacement in E134Q. This result contrasts with that of mutant E134D in which the simple curve shape is maintained although the level of activity and the pH optimum are changed. The appearance of the pH sensitivity curve for E134Q with an optimum and a pronounced shoulder at higher pH is an indication of other amino acid side chains contributing to a composite pH dependence. This will be discussed in more detail below.

In summary, replacements of Glu-134 affect the pH sensitivity of complex formation between rhodopsin and transducin. The sterically conservative replacement by the neutral amino acid Gln confers activity to the mutant pigment at a pH between 7.5 and 8.5. Neither rhodopsin nor the mutant E134D which possesses a sterically different but functional conservative amino acid replacement shows appreciable activity in this pH range.

Characterization of the pH Dependence of GTPγS-Induced Dissociation of Recombinant Pigments from Transducin. In a second set of experiments, the question of whether the mutations affected the pH sensitivity of GTPγS-induced dissociation of rhodopsin–transducin complexes was addressed. As explained under Materials and Methods, this could be tested by reducing the GTPγS concentration (100 nM) in experiments otherwise identical to those described above. The experiments with 100 nM GTPγS were carried out with aliquots of the same pH-adjusted rhodopsin–transducin mixtures used for the measurements in 5 μM GTPγS. Thereby, identical pH and reactant concentrations, except for GTPγS, were maintained. The activation rates obtained in 100 nM GTPγS are represented by open circles in Figure 4. An increase in the pH from 6 to 7.5 caused an increase in the rate of GTPγS-induced dissociation of rhodopsin from bound transducin (Figure 4a). This differs from the result obtained with high GTPγS concentration where the rates of complex formation already decreased above the optimum at pH 7 (solid circles). Therefore, complex dissociation at low GTPγS concentration (open circles) is favored at more alkaline pH as compared with rhodopsin–transducin complex formation. Above pH 7.5, the complex formation rate measured with 5 μM GTPγS becomes slower and approaches that of GTPγS-induced dissociation of rhodopsin measured with 100 nM GTPγS. Correspondingly, the GTPγS-induced complex dissociation no longer dominates the overall rate of the monitored accumulation of the GTPγS-loaded α-subunit of transducin. Instead, both reaction steps contribute to the observed kinetics in a more complicated manner not further analyzed in this study. Ultimately, a pH is reached at which the complex formation becomes so slow that it limits the activation rate even at low concentrations of GTPγS. As a consequence, identical rates were measured for both sets of experiments as shown by the coincidence of the traces represented by solid and open circles at pH 8.5.

A similar observation was made for the mutant E134D shown in Figure 4b (open circles). However, the reduced capability of this mutant to bind transducin prevented a significant accumulation of R*–transducin complexes. Therefore, a selective measurement of complex dissociation was difficult. Nonetheless, the effect of pH on complex dissociation

of mutant E134D is qualitatively the same as that observed with rhodopsin. An increase of pH in the pH range between 6.2 and 6.8 accelerated the dissociation reaction. The rate of complex formation was already low enough at pH 7 that it could not efficiently compete with the GTPγS-induced dissociation of transducin from R*. Correspondingly, the overlap between the E134D traces from "high" and "low" GTPγS concentration experiments occurred at a lower pH value (pH 8) than was observed for rhodopsin (pH 8.5), as described above.

The mutant E134Q exhibited a pronounced enhancement of catalytic activity in the alkaline pH range. This could be attributed to an accelerated complex formation rate as compared with rhodopsin. The accumulation of mutant E134Q bound to transducin in the nucleotide-free state is expected to occur more efficiently than with rhodopsin or mutant E134D. Therefore, GTPγS-induced complex dissociation should still determine the overall velocity of the fluorescence increase at pH values of 7.5 or more. In this pH range, the kinetics obtained in experiments on rhodopsin and E134D depended only weakly on changes in the GTPγS concentration (Figure 4a) or were too slow to respond at all (Figure 4b). The expected behavior of mutant E134Q was indeed observed as shown in Figure 4c (open circles). Again, an increase of the pH to more alkaline conditions than the pH optimum of complex formation (solid circles) accelerated complex dissociation, as was the case with rhodopsin. However, the pH at which GTPγS-induced dissociation became fast enough to compete with binding of the pigment to transducin was shifted to the alkaline outside the pH range tested. Thus, there was no overlap between the two traces in Figure 4c.

An important observation holds for each of the pigments. The pH sensitivity curves obtained with a 100 nM concentration of GTPγS could never be matched by a simple pH-independent scaling of the curves obtained in 5 μM GTPγS. This was obvious for the mutant E134Q since the bilobed form of the pH dependency curve of complex formation in 5 μM GTPγS was not found in experiments with 100 nM GTPγS. Even in the cases of rhodopsin and mutant E134D, where the shapes of the "high" and "low" nucleotide pH dependence curves were similar, a broadening to the alkaline side of the curves in 100 nM GTPγS was noted relative to the traces obtained in 5 μM GTPγS. Consequently, the curves obtained for rhodopsin and E134D in "low" and "high" concentrations of GTPγS could not be matched by a simple scaling factor in either case. The different pH responses of the underlying processes of complex formation and dissociation are best appreciated by comparing selected fluorescence time courses. As shown in Figure 5, a pair of pH values could be found for *each* recombinant pigment at which the effect of pH on the activation rate measured in 5 μM GTPγS was opposite to that obtained in 100 nM GTPγS. This result clearly demonstrates that the adjustment of the GTPγS concentration did indeed select for different reaction steps with different pH sensitivities. Due to the reduced activity of mutant E134D, this "kinetic separation" of both processes was less pronounced but still observable.

Taken together, the results obtained in the presence of 100 nM GTPγS show that GTPγS-induced dissociation is favored with increasing pH in all three recombinant pigments. The complex dissociation rate was accelerated for mutant E134Q in the entire pH range tested. The corresponding rates were similar for rhodopsin and mutant E134D. The amino acid replacements did not cause qualitative differences in the pH

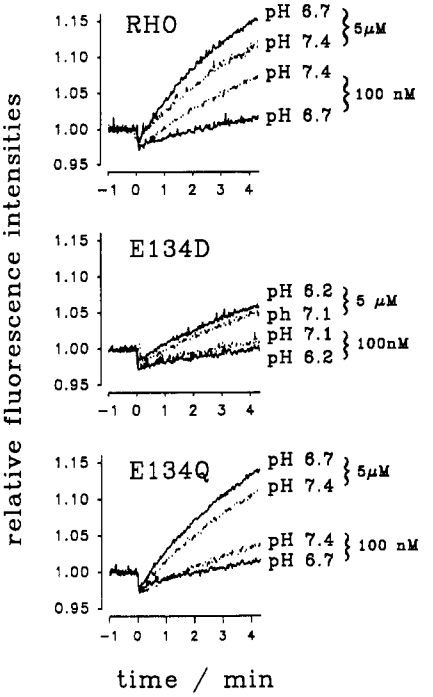

FIGURE 5: Time courses of transducin activation by rhodopsin, E134D, and E134Q. Individual fluorescence traces evaluated for generation of Figure 4 are shown at two pH values under conditions of 5 μM and 100 nM GTPγS concentrations as indicated. The results of separate experiments are superimposed. GTPγS was added at zero time to start the reaction. For each pigment, the results of experiments are shown at pH values where a change to more alkaline pH resulted in an increase in the complex dissociation rate (100 nM GTPγS) and a concomitant decrease in the receptor–transducin complex formation rate (5 μM GTPγS).

dependency of the GTPγS-induced dissociation step. Deviations were due to changes in the extent to which the complex dissociation actually dominated the overall reaction. This extent was smallest for mutant E134D.

Measurement of Light-Induced R Formation.* The salient effect of replacing Glu-134 was the ability of the mutant E134Q to bind transducin up to 15-fold faster than rhodopsin at high pH. In addition, a faster GTPγS-induced complex dissociation was observed for this pigment. It is known from native rhodopsin in disc membranes that the amount of R* formed upon illumination is a function of pH. Acidic pH was found to favor the MII (R*) conformation (λ_{max} = 380 nm) over the MI state (λ_{max} = 478 nm) in the absence of transducin (Mathews et al., 1963; Parkes & Liebman, 1984). Therefore, the enhanced rates caused by the substitution of Gln for Glu-134 could be due to a change in the pH sensitivity of R* formation such that a higher concentration of E134Q R* was present at pH >7.5 as compared with rhodopsin. The photobleaching difference spectra were measured for rhodopsin and E134Q in detergent micelles at pH 7.9 and 10 °C in the absence of transducin. Figure 6 shows that the absorption changes of rhodopsin and mutant E134Q were superimposable. Both traces show the formation of a pigment absorbing at 380 nm without any contribution of an MI-like form absorbing at 478 nm even at this alkaline pH, at 10 °C, in the absence of transducin. This finding agrees with the previously reported data showing that in dodecyl maltoside micelles the MII form of rhodopsin is favored over the MI state (Franke et al., 1992). Obviously, the mutation did not affect the predominance of a MII-like species.

Reversibility of pH Effects on Activation Rates. The striking difference between rhodopsin and mutant E134Q in

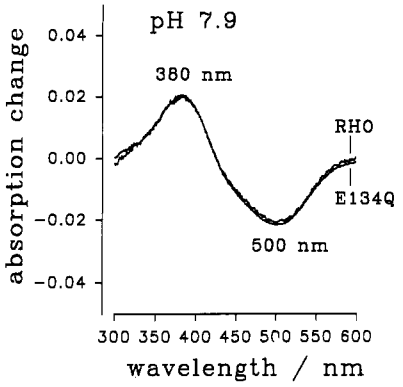

FIGURE 6: R* formation at pH 7.9 in detergent micelles. Photobleaching difference spectra are shown for rhodopsin and mutant E134Q. Rhodopsin and mutant E134Q were illuminated for 2 min with light of wavelength above 495 nm. Spectra were recorded at 10 °C in assay buffer in the absence of transducin.

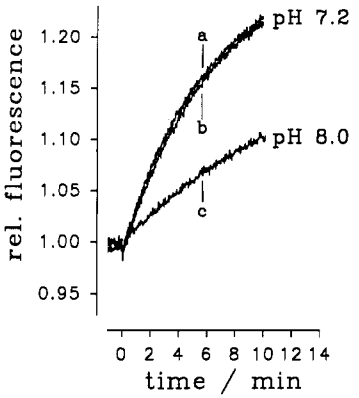

FIGURE 7: Reversibility of pH-induced changes of the rate of rhodopsin–transducin complex formation. Fluorescence time courses of transducin activation by rhodopsin were started by addition of 5 μM GTPγS at zero time. (a) Rhodopsin was converted to R* in the presence of transducin at pH 8. After 5 min, the pH was adjusted to 7.2 by addition of assay buffer of pH 6.1, and a fluorescence time course was recorded. (b) R* was generated at pH 7.2, and the fluorescence assay was carried out at the same pH. (c) R* was formed in the same solution assayed in (a), but the fluorescence time course was recorded at pH 8. Measurements were carried out at 10 °C.

the response of R*–transducin complex formation to pH could also be due to differences in R* stabilities at alkaline pH. In particular, the substitution of Gln for Glu-134 may have rendered the mutant pigment more stable in the detergent micelle as compared with rhodopsin. For example, rhodopsin could partially denature under basic pH conditions, reducing the effective amount of R*. To address this possibility, an experiment was carried out to show that the pH effects were reversible (Figure 7). A mixture of rhodopsin and transducin was illuminated at pH 8 under the described conditions of the assay. After 5 min, an aliquot of assay buffer (pH 6.1) was added to give a final sample pH of 7.2. GTPγS was added to start the fluorescence time course shown in Figure 7b. This reaction was compared to experiments in which R* was formed either at pH 7.2 or at pH 8 and assayed at the respective pH (Figure 7a,c). In particular, the pH 8 sample was taken from the same rhodopsin–transducin mixture (in the dark) used for the experiment in which the pH was readjusted to 7.2 shortly before the addition of GTPγS. The traces shown in Figure 7 clearly demonstrate that MII formed at pH 8 and incubated at this pH was capable of activating transducin after a pH change to 7.2 (Figure 7b) at the same rate as when it was formed and assayed directly at pH 7.2 (Figure 7a). Therefore, the lower rate obtained at pH 8 (Figure 7c) with the same sample used to generate the trace in Figure 7b is not caused by an irreversible denaturation of R*.

DISCUSSION

Earlier studies had shown that amino acid replacements at the highly conserved glutamic acid residue at position 134 in bovine rhodopsin could either decrease (E134D) or increase (E134Q) the rate of light-dependent transducin activation relative to that of native rhodopsin (Sakmar et al., 1989). In the present investigation, we have focused on specific reaction steps during the R*-catalyzed nucleotide exchange in transducin. The relative rates of formation and dissociation of complexes between photoactivated recombinant pigments and bovine transducin as a function of pH were measured. Complex formation rate is defined by the combination of rates of the necessary steps leading to a state in which transducin is bound to pigment and is able to bind GTPγS (Figure 1, line 3). As described under Materials and Methods, the contributions of these individual reaction steps to the overall transducin activation rate could be influenced by varying the GTPγS concentration. Measuring the reaction in either high or low GTPγS concentration provided information about the rates of R*–transducin complex formation and nucleotide-induced dissociation, respectively. Evidence for this "kinetic selection" of rate-determining steps was found in the different pH sensitivities obtained under both conditions as shown in Figure 5.

The pH dependency experiments were motivated by the fact that titratable surface charges may be involved in protein–protein interactions. Glu-134 can exist in a neutral, i.e., protonated, as well as in an unprotonated negatively charged state. Therefore, we expected the amino acid replacements at position 134 to affect the pH dependence of formation and/or dissociation of rhodopsin–transducin complexes. The pH sensitivity of transducin activation by recombinant rhodopsin has been reported previously using a GTPγ^{35}S binding assay under conditions which corresponded to the measurement of complex formation rates in this study (Cohen et al., 1992). The pH rate profile obtained in those experiments with rhodopsin in COS cell membranes was broader and slightly shifted to alkaline pH as compared to that measured in this study (Figure 4a, solid circles). The differences may be due to the absence of membranes in the fluorescence assay of detergent-solubilized pigments, thereby abolishing any local buffering capacity typically associated with biomembranes. The dodecyl maltoside detergent used did not contain titratable groups. Therefore, the pH of the bulk solution could be expected to be identical to that near the water-exposed surfaces of the recombinant opsin.

The salient result of the present study is the enhanced rate of complex formation at alkaline pH for mutant E134Q. Control experiments ruled out that either a shift in the MI/MII equilibrium or an effect on R* stability at pH 8 contributed to the differences in the complex formation rate. Therefore, the mutation affected the pH dependence of the rhodopsin–transducin binding process. This demonstrates that the fluorescence assay of detergent-solubilized mutant pigments allows the study of the interrelated influence of pH and amino acid replacement on specific *surface interactions* between pigment and transducin while the effective R* concentration remains constant. The interpretation of the pH dependencies is therefore different than that reported for the rate profiles in experiments carried out in the COS cell membrane system (Cohen et al., 1992). In the membrane assay system, the pH was shown to regulate the amount of active opsin conforma-

tions, comparable to the effect of pH on MI/MII concentrations in disc membranes and phospholipid vescicles (Mathews et al., 1963; Bennet, 1987, 1980; Parkes & Liebman, 1984; Hofmann, 1986). In disc membranes, it has been shown that the kinetics of transducin activation are indeed correlated with the rise time of MII and its equilibrium with MI (Kahlert & Hofmann, 1991).

The fluorescence assay monitored the accumulation of activated transducin rather than a rhodopsin–transducin binding equilibrium. For this reason, we ascribe the effect of the mutations on the activation rates with 5 μM and 100 nM GTPγS to alterations of the composite rate constants describing complex formation (Figure 1, steps 1–3) and dissociation (Figure 1, steps 3–5), respectively. The binding constants for the mutants are not necessarily changed by the amino acid replacements and cannot be inferred from these experiments. The more rapid formation of a complex between R* of mutant E134Q and transducin supports the involvement of Glu-134 in a prebinding process of the proteins. It has been speculated that this prebinding mechanism controls the efficiency with which rhodopsin–transducin collisions will eventually lead to the catalytic transition state (Hofmann & Kahlert, 1991). In addition to the effect on transducin binding, the substitution of Gln for Glu-134 also accelerated to a smaller extent the GTPγS-induced complex dissociation. Thus, both binding and GTPγS-induced dissociation were kinetically favored by a neutral amino acid side chain at residue 134. A possible interpretation of the data would suggest that Glu-134 interacts with transducin most efficiently in its neutral, i.e., protonated, state. A negative charge at this position would be prevented in mutant E134Q, thereby allowing higher activation rates. The slight shift to more acidic pH in the pH dependence curve of E134D agrees with the lower pK_a of Asp (pK_a $\sim$ 3.9) as compared to Glu (pK_a $\sim$ 4.2) in rhodopsin and supports an involvement of residue 134 in a titratable transducin-binding domain. However, the pH values which corresponded to the half-maximal activity of complex formation for rhodopsin and mutant E134D on the alkaline part of the respective pH dependence curves (Figure 4a,b, solid circles) cannot be identified with the pK_a values of Glu and Asp. Therefore, it is likely that other titratable groups contribute to a composite pH sensitivity of a transducin-binding region on rhodopsin. According to these data, either the net charge of this region is affected by pH-induced protonation changes of Glu-134, or this residue regulates a more alkaline pK_a of a different group without being titrated itself. Since we have demonstrated that MI is not present under the conditions of the assay, we exclude the involvement of His-211, found to regulate the pH sensitivity of the MII concentration (Weitz & Nathans, 1992). Surface-exposed residues are much more likely to contribute to the pH effects described in this study. Residues 143–151 of the second cytoplasmic loop, shown to be involved in transducin activation (Franke et al., 1992), contain an Arg and a Glu residue, and a total of 19 ionizable residues exist on the cytoplasmic face of rhodopsin. The participation of titratable groups other than Glu-134 is supported by the complex pH dependence of transducin binding obtained for mutant E134Q (Figure 4c, solid circles). If those parts of the curve which exhibit a steep pH dependence were to result from single amino acid residues undergoing protonation changes in the respective pH range, then a minimum of three residues would be needed to account for the structured curve. These data suggest that in native rhodopsin, Glu-134 tunes the resulting overall pH sensitivity of transducin binding to allow maximal rates at about pH 7. Since Glu-134 participates in the net charge regulation of a transducin-binding region in MII, one may hypothesize that its interactions with other residues or its protonation state are specifically altered upon photoactivation of rhodopsin.

In addition to accelerated complex formation, the rate of GTPγS-induced complex dissociation was increased in mutant E134Q. Consequently, Glu-134 is involved not only in binding transducin but also in its activation as judged by measurements of GTPγS-induced dissociation rates. Since it has been demonstrated *in situ* that the affinity of transducin for GTP is low as long as it is bound to MII (Kahlert & Hofmann, 1990), Glu-134 may be one of the residues that determines the mutual exclusion of MII and GTP as binding partners for transducin. Interestingly, the pH dependence of GTPγS-induced complex dissociation was not altered by the amino acid replacement. The pH-sensitive groups affected by mutations of Glu-134 seemed not to be titratable under conditions which allowed the accumulation of rhodopsin–transducin complexes. We interpret this result as a strong indication that the titratable surface domain of rhodopsin that includes Glu-134 becomes shielded from the bulk water phase once transducin is bound to rhodopsin. Therefore, we assume that Glu-134 is involved in interactions with transducin at the protein–protein interface, which ultimately affect the rate of GTPγS-induced complex dissociation. The pH dependence measured for the latter step may be due to the titration of the substrate GTPγS itself, favoring uptake by transducin at alkaline pH irrespective of the amino acid replacements. The titratable transducin-binding and activation region of rhodopsin is accessible only when the pigment is free in solution. Correspondingly, only under assay conditions that favor free R* do the mutations qualitatively affect the pH sensitivity of transducin activation. Since Glu-134 is highly conserved among G protein-coupled receptors, its described interaction with transducin may be a part of a general mechanism of G protein activation. Additional studies are underway to identify other amino acid residues involved in transducin activation.

ACKNOWLEDGMENT

We thank R. Franke and C. Min for helpful suggestions and J. Doherty of the RU Instrument Shop for expert spectrofluorometer modifications.

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