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Changing the Location of the Schiff Base Counterion in Rhodopsin[†]

Eugene A. Zhukovsky, Phyllis R. Robinson, and Daniel D. Oprian*

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254

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ABSTRACT: Rhodopsin and all of the vertebrate visual pigments have a carboxylic acid residue, Glu¹¹³, in the third transmembrane segment that serves as a counterion to the protonated Schiff base nitrogen of the chromophore. We show here that the counterion in bovine rhodopsin can be moved from position 113 to 117 without significantly changing the wild-type spectral properties of the protein. A series of double mutants were constructed where the Glu¹¹³ counterion was changed to Gln and an Asp residue was substituted for amino acid residues from position 111 to 121 in the third transmembrane segment of the protein. Only at position 117 can an Asp fully substitute for the counterion at position 113. The double mutant E113Q,-A117D has an absorption maximum at 493 nm which is independent of pH in the range 5.6–8.4 and independent of the presence of external chloride anions. An Asp at no other position tested in the third transmembrane segment can fully substitute for the Glu counterion at position 113. Partial substitution is observed for an Asp at position 120. Residues 113, 117, and 120 are expected to lie along the same face of an α -helix. These results suggest that the Schiff base nitrogen in rhodopsin is located between residues 113 and 117 but there is enough flexibility in the protein to allow partial interaction with an Asp at position 120. Position 117 is the same location of the counterion in the related biogenic amine receptors.

The visual pigment rhodopsin belongs to a large family of G protein-linked receptors that include adrenergic, cholinergic, and olfactory receptors (Dohlman et al., 1991; Buck & Axel, 1991; Hargrave & MacDowell, 1992; Khorana, 1992). These receptors are embedded in the lipid bilayer in the form of helical bundles composed of seven transmembrane segments. Rhodopsin contains an 11-*cis*-retinal chromophore covalently attached to the protein by means of a Schiff base linkage to the ϵ -amino group of Lys²⁹⁶, located in the seventh transmembrane segment (Bownds, 1967; Hargrave & McDowell, 1992). The spectral properties of the chromophore in rhodopsin are governed largely by interactions with the protein; in free solution a Schiff base of retinal has an absorption maximum at about 360 nm (Baasov & Sheves, 1986), whereas when bound to the protein the maximum shifts to 500 nm. Most of this shift can be accounted for by protonation of the Schiff base nitrogen (Zhukovsky & Oprian, 1989; Sakmar et al., 1989). The pK_a of the Schiff base nitrogen in rhodopsin is high; it has been estimated to be about 11 or 12 on the basis of the observation that the protein denatures at this pH without first losing the Schiff base proton (Radding & Wald, 1956). Typically, Schiff bases have pK_a s of about 6 (Baasov & Sheves, 1986). This stabilization of the protonated Schiff base in rhodopsin is a consequence of the juxtaposition of a negatively charged carboxylate residue from the protein, the Schiff base counterion (Honig et al., 1979; Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990).

Previous mutagenesis studies from our laboratory and others (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990) have shown that a Glu residue in the third transmembrane helix of bovine rhodopsin is the Schiff base counterion. This residue is Glu¹¹³. Changing Glu¹¹³ to Gln results in a dramatic decrease in the pK_a of the Schiff base nitrogen to

a value of about 6 (Zhukovsky & Oprian, 1989; Sakmar et al., 1989). Concomitant with deprotonation of the Schiff base nitrogen in the mutant E113Q¹ is a 120-nm blue shift in the absorption maximum to 380 nm. Both the high pK_a of the Schiff base nitrogen and the wild-type absorption maximum are restored upon changing residue 113 to Asp, which has a carboxylic acid side chain (Zhukovsky & Oprian, 1989; Sakmar et al., 1989).

Glu¹¹³ is conserved in all vertebrate visual pigments that have been sequenced to date from lamprey (Hisatomi et al., 1991) to human (Nathans et al., 1986). The biogenic amine receptors (including receptors for acetylcholine, dopamine, serotonin, histamine, and adrenergic ligands) also contain a highly conserved Asp residue in the third transmembrane segment (Dohlman et al., 1991; Oprian, 1992). Dixon and Strader and co-workers have shown that the Asp residue functions as a counterion to the charged substituted-ammonium-ion ligands in β_2 -adrenergic receptor (Strader et al., 1988). However, the Asp counterion is located at a different position in the biogenic amine receptors than is Glu¹¹³ in rhodopsin. As measured from the highly conserved Cys at the beginning of this transmembrane segment (Cys¹¹⁰ in rhodopsin), the Asp counterion in the biogenic amine receptors is in a position equivalent to Ala¹¹⁷ in rhodopsin, four residues further into the transmembrane helix than Glu¹¹³, roughly one helical turn away (Figure 1).

We show here that the Schiff base counterion in rhodopsin may be moved from position 113 to 117, the same position as the counterion in the biogenic amine receptors, with essentially no change in the pH-dependent spectral properties from those of the wild-type protein. We conclude from these data that the Schiff base nitrogen is located between these two positions in the third transmembrane helix such that a carboxylate side chain at either position will give rise to a high pK_a for the Schiff base.

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* To whom correspondence should be addressed.

¹ Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride. Mutant proteins are designated by single-letter code for the wild type, followed by the residue number, followed by the code for the new residue.

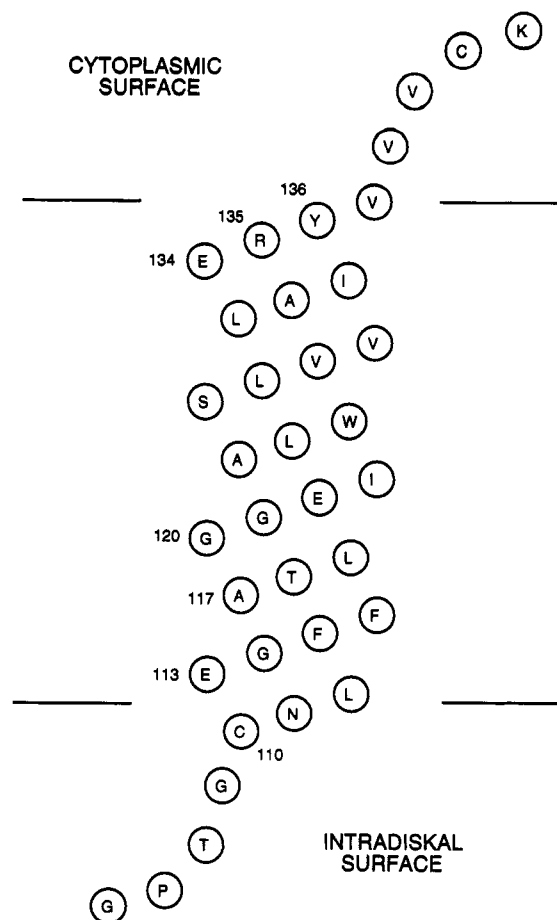


FIGURE 1: Schematic diagram of the third transmembrane α -helical segment of rhodopsin.

EXPERIMENTAL PROCEDURES

Materials. The monoclonal antibody rho 1D4, which is specific for the carboxyl-terminus of rhodopsin, has been described (Molday & MacKenzie, 1983). The antibody was purified from culture supernatant by precipitation with 50% saturated ammonium sulfate followed by ion-exchange chromatography on DEAE-Sephacel and then coupled to Sepharose 4B by established procedures (Cuatrecasas, 1970). Peptide I (DEASTTVSKTETSQVAPA, corresponding to the carboxyl-terminal 18 amino acids of rhodopsin), which competitively inhibits binding of rhodopsin to the rho 1D4 antibody (MacKenzie et al., 1984), was purchased from American Peptide Company, Inc. (Santa Clara, CA).

Bovine retina were from J. A. Lawson Co. (Lincoln, NE). Transducin was purified from retina according to the procedure of Wessling-Resnick and Johnson (1987), and then subjected to ion-exchange chromatography on DE-52 (Sigma) as described by Baehr et al. (1982). [35 S]GTP- γ -S and [γ - 32 P]-GTP were from New England Nuclear.

The detergent dodecyl maltoside was from Calbiochem, and CHAPS was from Sigma. The 11-*cis*-retinal was a generous gift from Peter Sorter and Hoffmann-La Roche.

Expression of the Rhodopsin Gene and Purification of the Protein. The design and chemical synthesis of the gene for bovine rhodopsin have been described previously (Ferretti et al., 1986). Expression of the gene in COS cells, reconstitution of the protein with 11-*cis*-retinal chromophore, and purification by immunoaffinity with the 1D4-Sepharose 4B matrix were also essentially as previously described (Oprian et al., 1987) with the following exceptions. COS cells were grown in 10% calf serum instead of fetal calf serum with little or no change

in yield of the protein. The cells were solubilized in 10 mM sodium phosphate buffer, pH 7.4, and 150 mM NaCl (PBS) containing 1% (w/v) CHAPS and 0.1 mM PMSF. After the protein was allowed to bind to the 1D4-Sepharose matrix, the beads were transferred to a 1-mL plastic syringe that had been plugged with glass wool. Unless noted otherwise, the matrix was washed 10 times by centrifugation in a clinical centrifuge with 1.0 mL each time of PBS containing 0.1% dodecyl maltoside. Rhodopsin was eluted with 50 μ M peptide I in the same solution.

Mutagenesis. Rhodopsin mutants were constructed in the synthetic rhodopsin gene using the method of restriction fragment replacement (Lo et al., 1984), as has been described (Zhukovsky & Oprian, 1989; Zhukovsky et al., 1991). Oligonucleotides were synthesized on an Applied BioSystems Inc. Model 380A DNA synthesizer.

Absorption Spectroscopy. UV/visible absorption spectra were recorded on a Hitachi Model U-3210 spectrophotometer that had been modified by the manufacturer for use in a darkroom. Data were acquired with the aid of an Everex System 1700 microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). All spectra were recorded on samples of 1.0-cm path length in thermostated cell holders with temperature maintained at 20 $^{\circ}$ C.

pH Titrations. For spectroscopic pH titration of mutant rhodopsins, the protein samples were prepared as described above in PBS or in 10 mM HEPES buffer containing 150 mM NaCl at higher pH (7.4–8.4). The pH was then adjusted by addition of calibrated aliquots of a solution of 1.0 M sodium phosphate, pH 5.1.

Effect of NaCl on the Spectral Properties of Rhodopsin Mutants. In order to determine the effect of external anions on the spectral properties of mutants, the proteins were purified initially in 10 mM sodium phosphate buffer, pH 6.1, with no NaCl present. After initial spectra were recorded, NaCl was added from a 3.0 M stock solution to give a final concentration of 150 mM. Where indicated, 10 mM HEPES buffer, pH 6.1, was used instead of phosphate as a control to show that phosphate did not substitute for the external anion under these conditions.

Reaction with Hydroxylamine. Solutions of the purified mutants were prepared in either PBS or 10 mM HEPES, pH 6.9, containing 150 mM NaCl. Prior to reaction with hydroxylamine, the pH of the protein solution was adjusted to 6.1 with a 1.0 M solution of sodium phosphate, pH 5.1. The reaction mixture was then brought to 20 mM in hydroxylamine concentration by addition of a 1 M stock of hydroxylamine hydrochloride that had been neutralized with NaOH. The reaction was carried out in the dark, and spectra were recorded at various times after the addition of hydroxylamine. Pseudo-first-order rate constants for the reactions were determined graphically from semilogarithmic plots of absorbance vs time.

Rhodopsin Activity. Rhodopsin activity was assayed in detergent solution by following the light-dependent activation of transducin using either GTP- γ -S binding (Wessling-Resnick & Johnson, 1987) or GTP hydrolysis (Neufeld & Levy, 1969) to monitor the reaction, as has been described (Zhukovsky et al., 1991). Rhodopsin was limiting (5 nM), and reaction rates were linearly proportional to rhodopsin concentration. Identical results were obtained with both assays.

RESULTS

Previous studies from our laboratory and others have shown that Glu¹¹³ in rhodopsin functions as a counterion to the protonated Schiff base nitrogen of the chromophore (Zhuk-

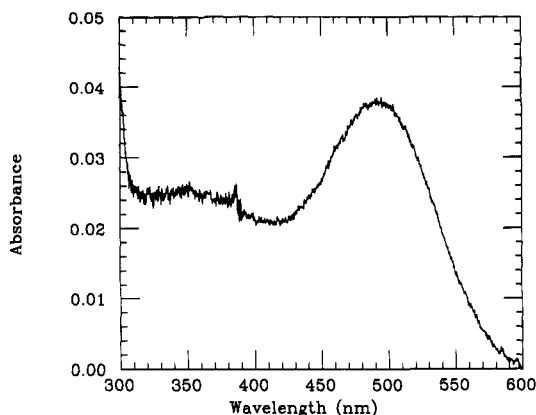


FIGURE 2: Absorption spectrum of the double mutant E113Q,A117D. The spectrum for the purified protein was determined in 10 mM HEPES buffer, pH 7.1, containing 150 mM NaCl and 0.1% (w/v) dodecyl maltoside.

ovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990). This conclusion is based largely on the observation that mutation of Glu¹¹³ results in a dramatic lowering of the pK_a of the Schiff base nitrogen. For example, the mutant E113Q as isolated at pH 7.4 is predominantly in the unprotonated form with an absorption maximum at 380 nm. Upon lowering the pH, the spectrum changes to that with a maximum at 493 nm. This change takes place with an apparent pK_a of about 6 (Zhukovsky & Oprian, 1989; Sakmar et al., 1989). The pH-dependent spectral behavior of the mutant is to be contrasted with that of wild-type rhodopsin. The absorption spectrum of wild-type rhodopsin has a maximum at 500 nm which is independent of pH in the range of pH 5–11 (Radding & Wald, 1956).

To explore the possibility that the counterion in rhodopsin could be positioned in the same location that it is found in β -adrenergic and the other biogenic amine receptors, we constructed a double mutant in which Glu¹¹³ was changed to Gln and Ala¹¹⁷ was changed to Glu or Asp. The mutant E113Q,A117E did not reconstitute with retinal chromophore (not shown). However, changing Ala¹¹⁷ to Asp resulted in a protein that did reconstitute with the retinal chromophore. The absorption spectrum for E113Q,A117D is presented in Figure 2. As isolated at pH 7.1 this protein displays a single long-wavelength absorption maximum of 493 nm, which is very similar to that of wild-type rhodopsin. Furthermore, this spectrum is unaffected by change of pH in the range 5.6–8.4 (Figure 3, top) and by a change in NaCl concentration in the range 0–150 mM (not shown), properties which are also characteristic of the wild-type protein. This behavior is very different from that of the single mutant E113Q, which shows two absorption maxima at 380 and 493 nm that are dependent on pH (Figure 3, bottom) and salt concentration (Nathans, 1990; Sakmar et al., 1991; Zhukovsky and Oprian, unpublished results). The dependence on salt reflects a requirement for an externally added anionic counterion in the E113Q mutant. We conclude from these data that the placement of Asp at position 117 in the double mutant fully compensates for the loss of the Schiff base counterion at position 113. That is, Asp¹¹⁷ is the Schiff base counterion in the double mutant E113Q,A117D.

The E113Q,A117D mutant activates transducin in a light-dependent manner similar to that observed for wild-type rhodopsin, as is shown in Figure 4. This was expected since the single mutant E113Q has wild-type activity (Sakmar et al., 1989). The fact that E113Q,A117D is fully active toward transducin suggests that the structure of the protein is not

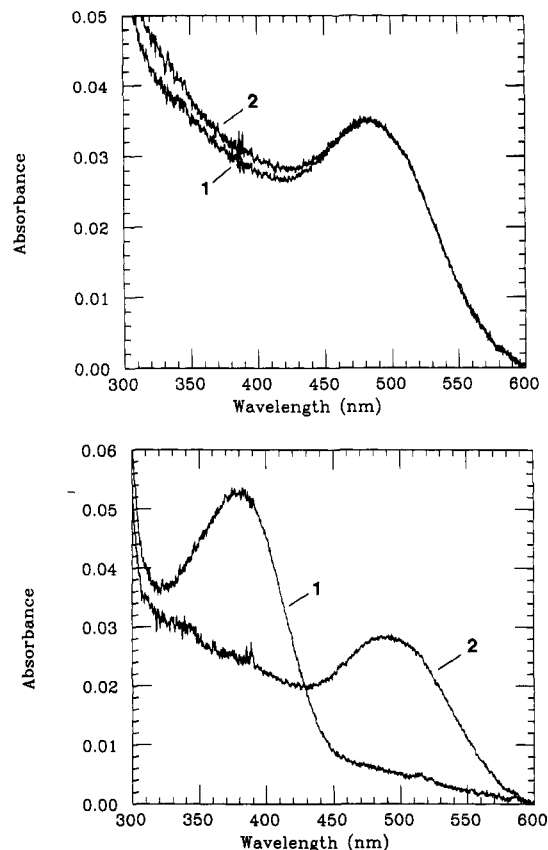


FIGURE 3: Comparison of the effect of pH on the absorption spectra of the mutants E113Q,A117D and E113Q. Top, E113Q,A117D; bottom, E113Q. The mutant proteins were purified in 10 mM sodium phosphate buffer, pH 8.4, containing 150 mM NaCl and 0.1% (w/v) dodecyl maltoside. After an initial spectrum was recorded, (curve 1), the pH was lowered to pH 5.6 by the addition of a solution of 1 M sodium phosphate, pH 5.1, and the spectrum was recorded again (curve 2). The spectra have been corrected for dilution.

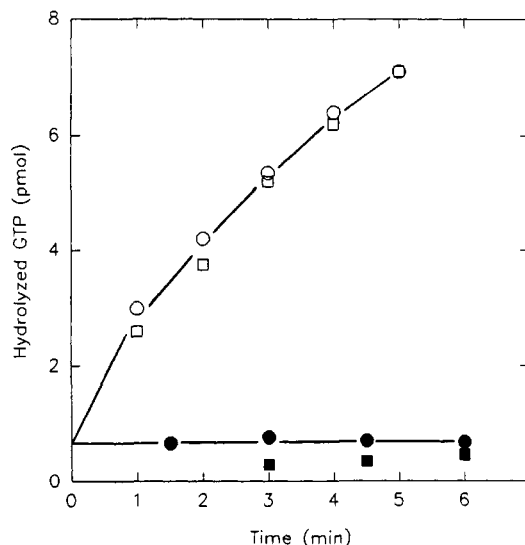


FIGURE 4: Light-dependent activation of transducin by wild-type rhodopsin and the mutant E113Q,A117D. Transducin activity was assayed by measuring the hydrolysis of [γ -³²P]-GTP. Circles, time course for the reaction catalyzed by wild-type rhodopsin purified from transfected COS cells; squares, time course for the reaction catalyzed by the mutant E113Q,A117D. Closed symbols, time points taken in the dark; open symbols, time points taken after exposure of the reaction mixture to light. Identical results were obtained from the GTP- γ -S-binding assay (not shown).

grossly altered by placing a carboxylic acid side chain at position 117.

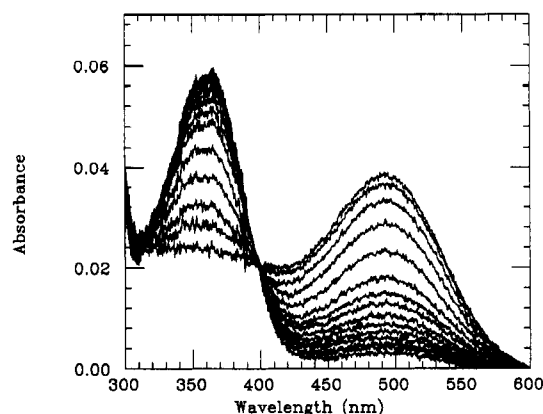


FIGURE 5: Reaction of the mutant E113Q,A117D with hydroxylamine. Spectra were recorded at various times after the addition of 20 mM hydroxylamine to the purified protein in 10 mM HEPES buffer, pH 6.1, containing 150 mM NaCl and 0.1% (w/v) dodecyl maltoside. Temperature was maintained at 4 °C. In order of decreasing absorbance at 500 nm, spectra were recorded at 0, 6, 20, 45, 80, 124, 152, 182, 212, 242, 272, 302, 332, 362, and 392 min after addition of hydroxylamine.

Despite the fact that the Asp at position 117 can substitute for the Glu¹¹³ in terms of the spectral properties of the chromophore, it did not substitute in terms of the resistance of the protein to attack by hydroxylamine. The double mutant E113Q,A117D differs from wild type in that it reacts with hydroxylamine in the dark, as is shown in Figure 5. The pseudo-first-order rate constant for this reaction is 0.005 min⁻¹ at 4 °C, pH 6.1, and a hydroxylamine concentration of 20 mM. E113Q also reacts with hydroxylamine in the dark (Zhukovsky & Oprian, 1989; Sakmar et al., 1989; Nathans, 1990). Wild-type rhodopsin does not react with hydroxylamine in the dark but reacts rapidly after exposure of the protein to light (Wald, 1968).

The single mutant A117D, in which a carboxylic acid side chain is placed at position 117 in a wild-type context instead of E113Q, was also constructed. A117D reconstituted with retinal chromophore poorly, but the pigment did have a long-wavelength maximum at about 475 nm, modestly blue-shifted relative to wild type (not shown). The blue shift is consistent with the idea that a second potential negative charge has been placed next to the Schiff base nitrogen in this mutant, stabilizing the ground state of the chromophore (Honig et al., 1976, 1979; Mathies & Stryer, 1976; Kakitani et al., 1985).

In addition to the E113Q,A117D double mutation discussed above, Asp substitutions were introduced at nine other positions in transmembrane segment III of mutant E113Q, from Asn¹¹¹ to Gly¹²¹. These double mutants are N111D,E113Q; L112D,E113Q; E113Q,G114D; E113Q,F115D; E113Q,F116D; E113Q,A117D; E113Q,T118D; E113Q,L119D; E113Q,G120D; and E113Q,G121D. Additionally, an Asp at position 113 was shown to substitute for Glu¹¹³ in previous work (Zhukovsky & Oprian, 1989; Sakmar et al., 1989). In stark contrast to Asp at positions 113 and 117, none of the other positions (with the possible exception of 120, discussed below) could substitute for loss of the Glu counterion at position 113. All of the other mutants either had spectra with a dominant 380-nm absorption maximum or were so poorly reconstituted with chromophore that a reliable spectrum could not be recorded. An example of a mutant that reconstituted well with chromophore is provided by N111D,E113Q in Figure 6 (curve 1). An example of a mutant that reconstituted poorly with chromophore is provided by L112D,E113Q in Figure 6 (curve 2).

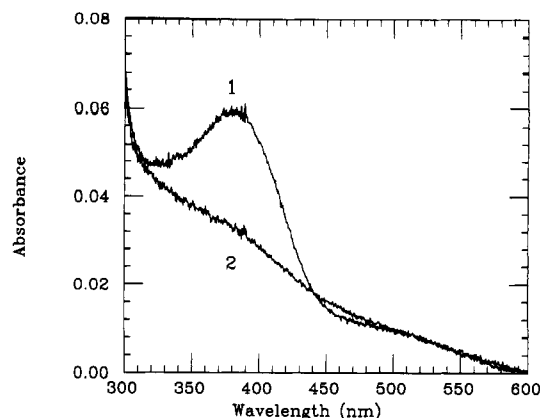


FIGURE 6: Absorption spectra of the mutants N111D,E113Q (curve 1) and L112D,E113Q (curve 2). The spectra are for the mutant proteins purified in 10 mM HEPES buffer, pH 7.1, containing 150 mM NaCl and 0.1% (w/v) dodecyl maltoside.

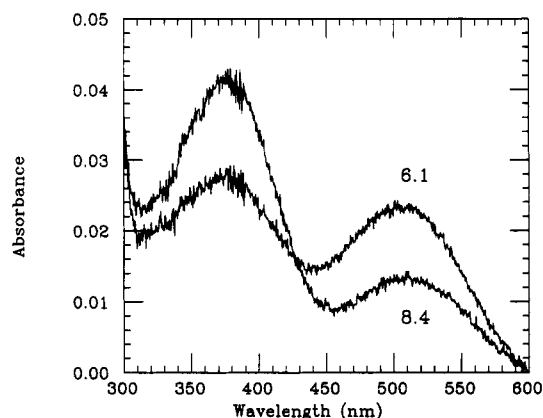


FIGURE 7: Absorption spectra of the mutant E113Q,G120D at pH 6.1 and 8.4. The mutant protein was purified in 10 mM HEPES buffer, pH 8.4, containing 150 mM NaCl and 0.1% (w/v) dodecyl maltoside. After the initial spectrum was recorded, the pH was lowered to 6.1 by addition of a solution of 1 M sodium phosphate, pH 5.1, and the spectrum was recorded again.

As shown in Figure 7, the mutant E113Q,G120D had somewhat anomalous behavior. The spectrum of this mutant at pH 6.1 was typical of the other double mutants that did not restore wild-type properties to the protein. However, when the pH was raised to 8.4, the E113Q,G120D mutant had a residual long-wavelength maximum (Figure 7), and this residual peak was not affected by salt concentration (not shown). In fact, the mutant behaves as if it is composed of two different protein species: one, amounting to about 75% of the population, typical of the counterion mutants having a Schiff base pK_a of about 6 and the other, representing about 25% of the sample, having spectral properties typical of the wild-type protein. The residual long-wavelength maximum is dependent on the presence of a carboxylate at position 120 since the double mutant E113Q,G120N behaves exactly like the single mutant E113Q (not shown). Therefore, an Asp at position 120 apparently can substitute partially for the counterion at position 113. The mutant E113Q,G120D activates transducin with wild-type specific activity and reacts with 20 mM hydroxylamine in the dark with a pseudo-first-order rate constant of 0.002 min⁻¹ at 4 °C and pH 6.1 (not shown).

DISCUSSION

The large family of G protein-linked receptors share a great deal of similarity in both structure and function. One of the

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Registry No. Glu, 56-86-0; Asp, 56-84-8.